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DEVELOPMENTAL CHANGES IN ALPHAFETOPROTEIN IN MOUSE YOLK SAC

by

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SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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Date April 8, 1981

To my wife Della, and my Mother and Father.

ABSTRACT

The yolk sac is an extraembryonic membrane which is formed at day 9.5 of gestation and is present until birth. Although the structure and function of the yolk sac during gestation have been studied, there is very little biochemical information available. I have investigated the dynamics of synthesis of various macromolecular species in the yolk sac during the latter half of gestation (day 11.5 to 18.5).

In my initial studies, the synthesis of DNA, RNA and protein were all shown to decline drastically during this period. These changes were accompanied by a reduction in growth and deterioration in structure near birth (83). These biochemical changes are consistent with the hypothesis that the yolk sac is undergoing tissue senescence (195).

Changes in the synthesis of individual proteins were analyzed using SDS and two dimensional polyacrylamide gel electrophoresis. During the decline in total protein synthesis, most proteins retained the same relative rates of synthesis. However, four proteins showed an increase in relative synthesis and constituted the major fraction of the protein synthesis at day 16.5. These four proteins also proved to be the main proteins secreted by the yolk sac and two of the secreted proteins were found in the amniotic fluid. The selective synthesis and secretion of these proteins is thought to be an example of coordinate gene expression.

The two main proteins synthesized and secreted at day 16.5 were identified as alphafetoprotein (AFP) and transferrin (Tf). Quantitative analysis of AFP synthesis by immunoprecipitation showed that the relative rate of AFP synthesis increased from 3% at day 9.5 to 22% at day 15.5

and then decreased. This change correlates with the relative proportion of AFP mRNA during this period of gestation.

In my initial experiments designed to study the stability of AFP mRNA, I used the inhibitor of transcription, α-amanitin. These studies indicated that AFP mRNA and the mRNA's of several other proteins were very stable. Further analysis using pulse and chase conditions revealed that the AFP mRNA is 10 times more stable than the total poly $(A)^+$ RNA. This could be an underlying mechanism in regulating the relative amount of AFP mRNA in the yolk sac during gestation.

AFP was the major secretory protein constituting about 50% of the total protein secreted from day 11.5 until birth. The electrophoretic and isoelectric species of AFP change during gestation, apparently due to the increased addition of sialic acid. The inhibition of N-linked glycosylation by tunicamycin did not affect AFP secretion, indicating that this type of glycosylation is not required for secretion. The yolk sac would be a good system to study factors that may be involved in the synthesis, secretion and modification of AFP.

The yolk sac from day 11.5 to day 18.5 therefore represents a system with which to study the metabolic events for functional specialization and tissue senescence.

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ABBREVIATIONS

A_{260}	light absorbance of a solution in a 1 cm light path at 260 μm (for example)
AFP	alphafetoprotein
BSA	bovine serum albumin
cDNA	complementary DNA
EDTA	disodium ethylenediamine tetraacetate
E.R.	endoplasmic reticulum
$7\ ^{Me}G$	7 methyl guanosine
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
ICM	inner cell mass
mRNA	messenger RNA
M.W.	molecular weight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCA	perchloric acid
PMSF	phenylmethylsulfonylfluoride
RNP	ribonucleoprotein
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Tf	transferrin
tRNA	transfer RNA
Tris	tris (hydroxymethyl) amino methane
VLDL	very low density lipoprotein

CHAPTER I

INTRODUCTION

The mechanisms of cell differentiation during embryonic development are of great biological and medical interest. To understand the attainment of structure and function by various embryonic tissues, the biochemical events and their changes during gestation must be studied. The molecular mechanisms which control these biochemical events must then be examined to obtain an insight into the differentiation process.

In this work, I have investigated gene expression and possible control mechanisms in the mouse yolk sac during the last half of gestation. Particular emphasis has been placed on the expression of the alphafetoprotein (AFP) gene, since AFP is the major protein synthesized by the yolk sac (241).

A. Biology of the Yolk Sac

1. Structure

During the gestational period of day 11.5 to 18.5, the mouse fetus is surrounded by three membranes (Fig. 1). The outer membrane is the parietal yolk sac which ruptures by day 13.5 and retracts to the placental cap. The visceral yolk sac is situated between the parietal yolk sac and the amnion which is the membrane nearest the fetus. The visceral yolk sac is continuous with the chorio-allantoic placenta, forming a complete sac around the fetus from day 10.5 of gestation to birth. Two unique features of the yolk sac

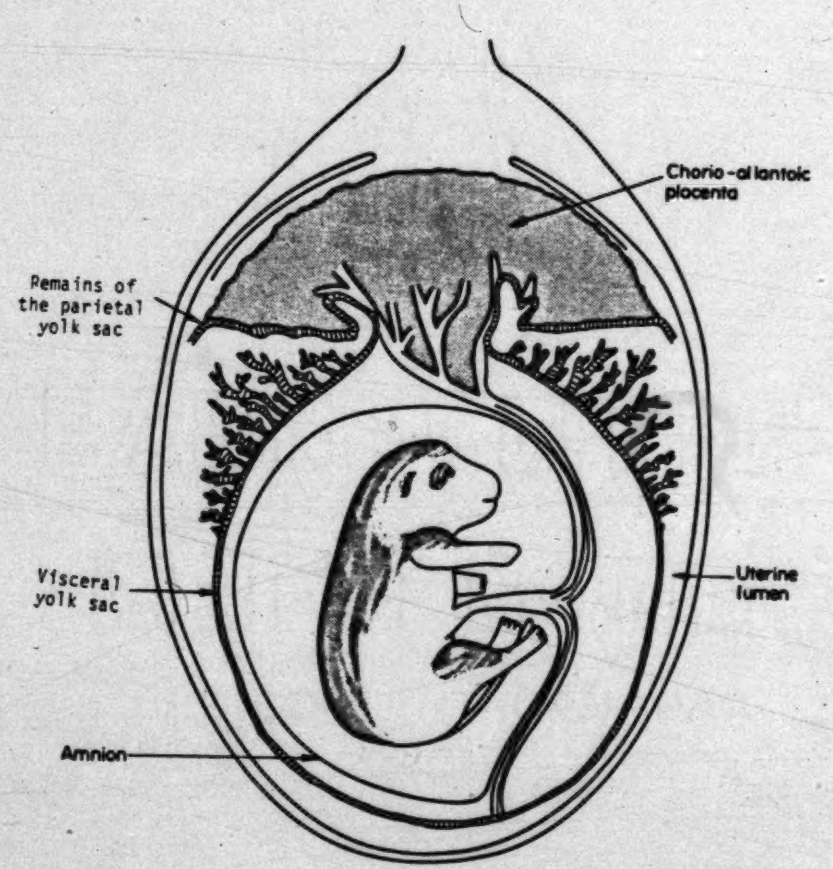

Chorio-allantoic placenta
Remains of the parietal yolk sac
Visceral yolk sac
Uterine lumen
Amnion

Figure 1. Schematic diagram of the fetal mouse and its membranes at day 13.5 of gestation (100).

are the presence of a large number of vitelline vessels which carry fetal blood (242) and the ridges surrounding the site of yolk sac-placenta attachment (195). The yolk sacs of rats and mice seem to be identical as far as structure and function are concerned and information gleaned from either will be discussed without referring to the species.

The morphology and ultrastructure of the yolk sac have been well studied (Fig. 2) (36, 116, 153, 182, 242). The yolk sac is composed of three cellular layers separated by two basement membranes (Fig. 2). The visceral endoderm cells initially face the parietal yolk sac and then after the parietal yolk sac ruptures, the uterine lumen. These cells rest upon the visceral basement membrane which separates these cells from the mesodermally derived cells underneath. The mesothelium is a thin layer of cells which borders the amniotic cavity and rests on the serosal basement membrane. Between the two basement membranes are the mesenchymal cells which include the vitelline vessels.

Ultrastructural studies of the yolk sac have concentrated mainly on the endoderm cells (36, 116, 153, 182). Figure 2 describes many of the interesting features of these cells. They are simple columnar cells. The outer surface consists of numerous microvilli which vary in length and are, in many cases, branched. In the region between these projections, invaginations form which are believed to be connected to the membrane bound compartments seen just below the cell surface. This system of membrane bound vacuoles and canals has been called the "cannicular system" (116) or "tubulo-vesicular system" (36). This system is found in many types of cells involved in protein absorption

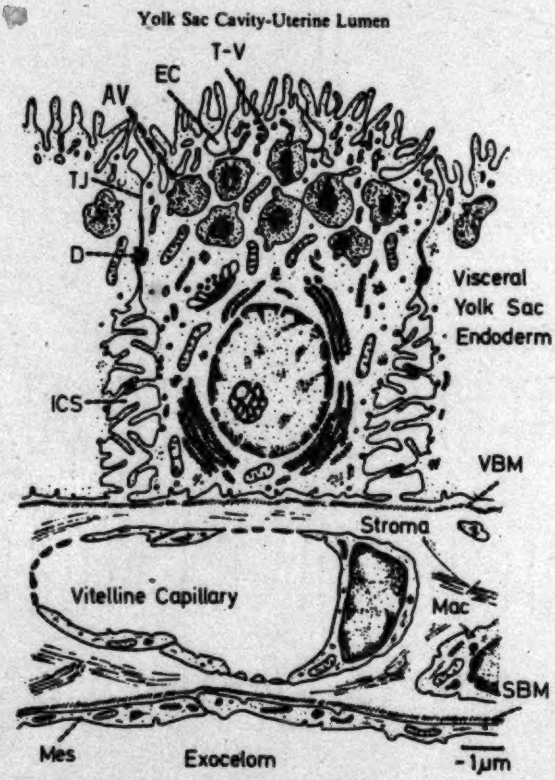

Yolk Sac Cavity-Uterine Lumen
T-V
EC
AV
TJ
D
Visceral Yolk Sac Endoderm
ICS
VBM
Stroma
Vitelline Capillary
Mac
SBM
Mes
Exocelom
1μm

Figure 2. Semischematic drawing of a segment of rodent visceral yolk sac. The fine structure features illustrated are typical of the yolk sac during the last third of gestation in these species. Abbreviations: AV, apical absorptive vacuole; D, desmosome; EC, endocytotic channel; ICS, intercellular space; Mac, macrophage; Mes, mesothelium; SBM, serosal basement membrane; TJ, tight junction; T-V, apical tubulo-vesicular system; VBM, visceral basement membrane. Approximate scale as indicated (36).

and is thought to be intimately involved in pinocytosis. The nuclei in these cells are found basally as are the E.R. and Golgi apparatus. Lipid and glycogen storage granules are also found in the lower region of the cell. Tight junctions between the lateral edges of the endoderm cells near the outer edge of the yolk sac form an impenetrable barrier between the intercellular space and the uterine lumen (36). Desmosomes, which are thought to join cell membranes together, are found along the lower, lateral regions between endodermal cells. In this region the cell membranes are twisted and the intercellular region is much larger than near the outer edge. These spaces contain a granular material which is optically similar to material in the Golgi apparatus (116).

The endoderm cells sit on the visceral basement membrane which contains collagen and possibly aminoglycans (5, 242). Underneath this layer is the mesenchymal layer which is mostly connective tissue and contains the vitelline vessels. The inter-basement membrane region also contains collagen fibers and macrophages. The serosal basement membrane constitutes the inner substratum layer. Differences in the structural components of the two basement membranes have been shown, although this does not preclude similar function (5, 242).

The inner cellular layer bordering the amniotic fluid consists of a simple layer of squamous cells derived from the mesoderm. These cells have not been well studied but do not have any unique structural features that suggest anything other than a structural function.

2. Development of the Yolk Sac

Formation of the yolk sac is a dynamic process which is intimately linked with the development of the embryo. The gestational period of the mouse is usually 20 to 21 days. Fertilization of the ovum usually takes place between midnight and 2:00 a.m. and successful mating is shown by the presence of a vaginal plug the morning after (171). The morning of the appearance of the vaginal plug is denoted as day 0.5 of gestation.

The early events of embryogenesis are described in detail by R. Rugh and Snell and Stevens (171, 193). To briefly describe these events, the newly fertilized ovum goes through a number of cell divisions as it moves from the upper end of the oviduct to the uterus. By day 3.5 a clump of 16-32 cells called a morula is formed. At this stage all the cells appear to be identical. During the next several days, a cavity forms inside this group of cells and the blastocyst is formed. The cells around the outside undergo the first visible differentiative step to form the trophectoderm (170). The cells on the inside are called the inner cell mass(ICM). On day 4.5-5.0, the blastocyst implants into the uterine wall. During this process, the layer of ICM cells that face the blastocoelic cavity undergo the second differentiative step to form the primitive endoderm (170). The primitive endoderm gives rise to the endoderm layer of both the parietal and visceral yolk sacs (53, 57, 66). From day 5 to 7, the inner cell mass grows into the blastocoelic cavity forming the egg cylinder (171, 193, 170, 124).

The egg cylinder can be divided into the embryonic portion which will eventually form the fetus and the extraembryonic portion which will form the placenta and other fetal membranes. From day 7-8, the mesoderm and definitive endoderm layers appear within the primitive streak region of the embryonic ectoderm. The mesoderm grows forming a layer between the primitive endoderm and the ectoderm and eventually reaches up to the extraembryonic region where it takes part in tissue formation (66, 89, 90, 124, 193). From day 9 until birth, organogenesis takes place and the embryo is now called the fetus.

By day 10, the yolk sac has become a separate tissue (52). At this stage, the vitelline vessels are already present. The development of this vascular system begins at day 8 when the blood islands are formed in the newly differentiated mesoderm (193, 131). The endothelial cells differentiate from the mesoderm to form the initial fetal blood vessels (vitelline vessels). The embryonic blood cells appear in the blood islands at this time. These blood cells are unique in that they retain their nucleus throughout their life span, although it appears to be inactive (131). They are present in the circulatory system until about day 12 and 13, during which time they synthesize the fetal globins α, X, Y, and Z (131,18). Moore and Metcalf believe that the yolk sac is the site of formation of the myeloid and hemopoetic cell precursors which eventually populate the adult tissues (bone marrow, thymus and spleen) (139). Recently it has been shown that a set of immature T cells is present at day 8 and that they disappear by day 17, at which time the spleen and thymus contain such cells (38).

To summarize the development of the yolk sac, the visceral endoderm cells are formed from the primitive endoderm cells which differentiate quite early during embryo development. The mesoderm portions of the yolk sac form much later and eventually give rise to the mesenchyme, mesothelium and the blood islands. During the organogenesis stages (day 9.5 to birth), the yolk sac does not appear to undergo any further morphological changes. However, as will be shown in Chapter III, changes in metabolism take place during this period.

3. Function of the Yolk Sac

The yolk sac is important for the normal development of the fetus (157, 166). Since the yolk sac surrounds the fetus during its development, it determines, to some extent, the environment within which the fetus develops. It also acts as a barrier between mother and fetus.

The fetus is suspended in the amniotic fluid which is believed to act as a shock absorber protecting the fetus from mechanical injury (157). Furthermore, the yolk sac appears to protect the fetus from toxic compounds present in the mother (240), by providing an impermeable surface formed by the tight junctions between the endodermal cells (36). These cells appear to be very absorptive but also contain many degradative enzymes, so few compounds are moved across this cell layer intact (195, 190, 237). The yolk sac also accumulates toxic material, e.g. trypan blue, in its apical portion and does not apparently transport them to the fetus (182).

The absorption of maternal compounds by the yolk sac has been well documented and two important functions have been suggested (153, 157, 237). The absorption of proteins, lipids and other maternal molecules takes place and these are then degraded in the lysosomes (237) or stored in vacuoles (203). These are eventually released into the vitelline vessels and used in fetal metabolism. Ultrastructural studies of the endoderm cells show that lipid and glycogen granules accumulate in the basal end of the cell (195). The storage of such energy rich compounds suggests that the yolk sac is behaving in a similar fashion to the liver in the adult.

The transfer of passive immunity from mother to fetus is the second major function associated with active absorption by the endoderm cells (29). The transfer of immunity to the fetus is very important for the survival of the animal after birth. Maternal antibodies are found in the fetal serum and are transferred preferentially when compared to other maternal proteins (8, 236). The yolk sac is believed to be the sole origin of such transfer as the placenta is not able to transfer IgG molecules (208, 234). The selective transfer is governed by the endodermal cell layer (234, 191). During the transfer, IgG molecules seem to be resistant to proteolysis when compared to other proteins (238). The selectivity of IgG transfer has been shown to be due to receptors in the invaginated region of the endodermal cell surface (56, 235). These receptors are specific for the Fc portion of the IgG molecule (41, 181, 215, 216).

The rat yolk sac has been used by Lloyd and co-workers as a model system to study pinocytosis (50, 51, 167). Essentially, their work shows that molecules can enter the yolk sac in two modes: (1) liquid phase, or (2) membrane bound. Molecules which can bind to the membrane are taken up more efficiently than those in the liquid phase. The process of pinocytosis requires energy and calcium and involves the microtubular system of the cell. The pinocytosis of marker protein by the yolk sac is severely inhibited by increased level of cAMP, although the physiological reason for this effect is not understood (31).

A model has been proposed to account for the differential transport of maternal proteins (29, 238). All proteins are taken up by pinocytosis, most in the liquid phase and others such as IgG which have specific receptor,are bound to the membrane. These vesicles eventually form or fuse with lysosomes and the proteins in the liquid phase are digested. The Fc receptor bound IgG is protected from degradation. An alternative possibility, suggested by William and Ibbotson (238), is that the IgG bound vesicles do not become involved with lysosomes and are therefore transported intact. Mechanisms of receptor mediated endocytosis or pinocytosis are presently receiving a great deal of attention (45, 76, 93, 239).

The mechanism of vesicle movement through the cells is not well understood. When these vesicles reach the basal membrane, their contents are released into the extracellular space by reverse pinocytosis (236, 238). Once released, the proteins diffuse into the mesenchyme where they are taken up by the vitelline vessels and transported to the fetus (29).

In these latter experiments when the vitelline circulation was stopped, the transfer of IgG was inhibited. Other effects of this procedure were the deterioration of the yolk sac and a decrease in the volume of amniotic fluid (29, 62). These observations agree with the work of Payne and Deuchar (157) which showed that the yolk sac is involved in the regulation of extraembryonic fluids. The content and the amount of amniotic fluid are found to change during the last half of gestation (166). The changes in the type of amniotic fluid protein reflect those found in the fetal serum 24 hr previously (79, 80). This would suggest that the majority of the proteins released by the yolk sac enter the vitelline fluid first and then enter the amniotic fluid although some possible exchange may take place directly across the mesodermal layers (116).

The importance of the yolk sac to the normal development of the fetus has been shown by the effects of toxic agents on the yolk sac. The earliest example of this was the teratogenic effect of trypan blue injected into the mother (182). The dye accumulates in the visceral endoderm cells and is believed to cause its malforming effect by disrupting the nutritional function of the endoderm cell. Similar results have been obtained using antibody made against whole yolk sacs. These have been shown to cause death, growth retardation and malformations and have been shown to localize only in the endoderm cells of the visceral yolk sac (101, 144). The underlying mechanism for these effects is not understood at present.

These results would suggest that any immune attack by the mother on the yolk sac should cause a large proportion of stillbirths or malformations. The visceral yolk sac is exposed directly to the uterine cavity from day 12 to birth and isolated endoderm cells have been shown to contain H-2 and non H-2 antigens that can be detected by an allogeneic immune response (100). Recently the exposed surface of the endoderm layer has been shown not to contain these antigens (156). This may explain why there is no maternal response to the yolk sac.

An interesting feature of the yolk sac endoderm cells is that the maternal X chromosome is preferentially active while the mesodermal cells, as all fetal tissues, show random X inactivation (224, 233). Recently it has been shown that the trophoectoderm also has the maternal X active (63). Both the trophoectoderm and visceral endoderm arise during the first steps of embryonic differentiation and are in intimate contact with the maternal environment during gestation. A relationship between these observations has been suggested (63, 224, 233). The visceral yolk sac would make a useful system to study the mechanisms of X chromosome inactivation.

In our laboratory, we are interested in the expression of the AFP gene and its control in the yolk sac. Early work had shown that the yolk sac of the rat and mouse synthesize AFP (71, 241). The synthesis of AFP in the mouse embryo begins soon after the formation of the visceral endoderm (52). The yolk sac endoderm is believed to be responsible for the synthesis of serum proteins (52,244). The synthesis of AFP and other serum proteins is believed to be an important function of the yolk sac.

B. AFP

1. Biology

AFP is a serum protein present during fetal development (for reviews see 1, 6, 88, 175, 187). After birth serum levels of AFP decrease markedly and normally remain low throughout adult life. However serum AFP becomes elevated during certain pathological conditions, of which hepatocarcinoma and teratocarcinoma are of most interest (1).

AFP is synthesized in a number of different species ranging from sharks to all mammals studied thus far (73, 74, 30, 185). During mammalian fetal development, the yolk sac synthesizes AFP and other serum proteins (72, 71, 241). The human yolk sac atrophies after three to four months gestation and ceases to synthesize AFP (72). The liver is the other major organ which synthesizes AFP. The hepatocytes derived from the endoderm are responsible for the synthesis of AFP as well as albumin (4, 43). AFP is secreted by these tissues and is present in high levels in the fetal serum and amniotic fluid. These high levels in turn lead to increased levels in the maternal serum (175, 185). This latter relationship is of clinical interest and will be discussed below. The AFP concentration in adult serum is very low, e.g. human, 5-25 ng/ml. The normal levels vary between species and between different strains of the same species (133, 151, 162). The appearance of AFP in adult serum is indicative of hepatocarcinoma (1), teratocarcinomas containing yolk sac elements (114, 206) and non-malignant liver disease (6).

The presence of high concentrations of AFP during fetal development strongly suggests that it has an important role. Murgita and Tomasi have suggested that it is involved in protecting the immunogenic fetus by inhibiting the maternal immune response (141). In these studies, AFP inhibited both humoral and cell mediated immunity as determined by *in vitro* assay (141, 142). These experiments were done using the mouse system and similar results have been obtained with human AFP (120). However, these results could not be duplicated by S. Sell *et al.* (186, 189). The reasons for these differences are not clear (68, 210). There is no evidence that AFP is immunosuppressive *in vivo* (64, 186, 189), indicating that AFP may not be of physiological importance as an immunosuppressive agent (68).

The discovery that AFP can bind estrogens very tightly suggested another possible physiological function (219). Of all the species tested, only rat and mouse AFP have been shown to bind estrogens (149, 220). AFP binds specifically to estradiol and estrone and not to testosterone or progesterone (13). The binding site is an intrinsic part of the protein although changes in the carbohydrate portion may alter the binding slightly (14, 194). AFP has been recognized as the estradiol binding protein found in the brain. Several investigators have suggested that it is involved in protecting the fetal brain from maternal estrogens during sexual differentiation (12, 135, 138).

However, the fact that human AFP does not bind estrogens suggests that this may not be the primary function of AFP. Analysis of material bound to human AFP revealed that it consists mainly of fatty acids (155). Furthermore a fatty acid fraction was also present in pig, rat and cow AFP (91, 95, 221). Bilirubin,which usually binds to albumin can also bind to human, rat and bovine AFP (25, 91, 223). Analysis of the fatty acid fraction revealed that the long chain poly-unsaturated fatty acids, especially arachidonate, are preferred over short chain, saturated fatty acids (35). These types of essential fatty acids are required for fetal nutrition and especially for development of the nervous system. They are also precursors of prostaglandins and may therefore play an important role in fetal development (35, 91, 221).

Comparison of the components bound to both albumin and AFP revealed that similar types of fatty acids are present but that their affinity for AFP is higher (95, 155). This led to the proposal that AFP may be acting as a fetal albumin and one of its main functions is to transport hydrophobic compounds throughout the fetus (91, 95, 221). Analysis of this binding site on AFP and albumin showed that they are functionally and geometrically similar (25).

These results suggest that the binding of estrogen is an *in vitro* artifact. Furthermore, Aussel *et al.* showed that the increased levels of AFP found in rats treated with carbon tetrachloride had no effect on serum estrogen levels (15). The fatty acid fraction has a higher affinity for rat AFP than do the estrogens and this might explain why estrogens are not found on purified AFP (221). Notwithstanding the effect of AFP on the sexual differentiation of the brain, the nutritional value of the AFP bound fatty acids may be of greater importance. The presence of AFP in the brain may also be fortuitous, as all major serum proteins are present and these may enter non-specifically during fetal development (212).

2. Chemistry

The structure of AFP has been the subject of many studies (review, see 175). In general, AFP from different species appears to be very similar. AFP consists of a single polypeptide chain with an attached carbohydrate portion. The molecular weight of AFP ranges from 69,000 to 74,000 depending on the species. The carbohydrate portion constitutes approximately 4% of the molecular weight and consists of mannose, galactose, N-acetylglucosamine and sialic acid. The amino acid compositions of AFPs from different species have been determined and they are similar. The similarities between AFPs of different species are also shown by their ability to cross react immunologically, although some differences have been noted between rodent AFP and other species (146).

Since the proposal that AFP and albumin may have a similar physiologic role, a comparison of their structures has been made. While the amino acid compositions of albumin and AFP are very similar, albumin does not contain any carbohydrate (175, 176). There is no immunologic cross reaction between these proteins in their native state; however, antibodies to denatured albumin and AFP do cross-react (172). AFP has been partially sequenced and this sequence compared to albumin (160, 173). The results indicate that there is approximately 50% homology between the middle and C terminal portions of the proteins but none at all at the N terminal end. However, as the complete sequences have not yet been determined, the true extent of the homology is not known. Furthermore, complementary DNA probes made to albumin and AFP mRNA's do not hybridize to their heterologous mRNA's (177). However, nucleotide sequence of the 3' end of the two mRNA's showed approximately 57% homology (123). The hypothesis that AFP and albumin originate from the same ancestral gene, as suggested by partial sequence homology, has yet to be substantiated.

During the structural studies, it was revealed that AFP was heterogeneous with respect to electrophoretic mobility and lectin binding (192). The reason for this heterogeneity has been intensively studied in mouse, rat and human but no definite conclusions have been drawn. The heterogeneity exhibited by mouse AFP is probably the least complex in that the electrophoretic variation has been found to be due to differences in sialic acid content (80, 246). The heterogeneity with respect to lectin binding is probably due to the different patterns of glycosylation by the liver and yolk sac (174). The heterogeneity of rat and human AFP is more complex in that it has been shown to be due only in part to carbohydrate changes (106,121,228). The binding of the fatty acid fraction to human AFP causes changes in the isoelectric point (155). The functional differences caused by the heterogeneity are not well understood.

3. Clinical

An increased level of AFP in adult serum is indicative of disease (6). Screening of high risk populations for hepatocarcinoma or endodermal sinus tumors for AFP serum concentration has been proposed. An increase in AFP levels occurs early in these cancers and early detection of cancer leads to a better prognosis (3, 134, 187). Wide scale screening has not been done as the incidence of these tumors is low, false positives due to non-malignant disease are present,and not all hepatomas synthesize AFP (6, 175). The monitoring of AFP serum levels has been found to be useful in following the disease once it has been detected. As AFP concentration is believed to reflect tumor mass, reductions would indicate that treatment is destroying the tumor. Relapse of disease may also be detected earlier (67, 102).

Recently, actual tumor mass has been visualized by scintillation photography of ^{131}I-labelled antibodies for AFP which bind to AFP-synthesizing tumors (107, 111).

Certain non-malignant liver disorders are associated with elevated AFP levels. Chronic hepatitis and cirrhosis of the liver show slightly increased levels (2, 82). Furthermore, elevations in serum AFP are found in conjunction with hereditary tyrosinemia and ataxia telangiectasia (22, 225). These higher levels are believed to be indicative of liver regeneration.

Screening of pregnant women's serum for increased AFP concentration has proven to be an excellent method to monitor the health of the fetus (84). The serum levels of AFP are high in cases of open neural tube defects, intrauterine death and congenital nephrosis (32, 84). To confirm that high serum AFP concentrations indicate a fetal abnormality, the amniotic fluid should be checked for increased AFP and for the presence of other markers of fetal distress (165).

Attempts have been made to treat AFP-producing tumors with autologous or heterologous antibody. Initial *in vitro* studies proved to be successful (10, 217, 232), while studies using transplantable AFP-producing hepatomas were not (133). The latter results showed that serum AFP levels decreased but there was no effect on tumor growth. This has been confirmed in a patient with an AFP producing hepatoma (111).

4. AFP Gene Expression and its Regulation

The control of AFP expression is interesting from two aspects: (1) control of gene expression during normal differentiation, and (2) aberrant gene expression during neoplastic transformation (4). These have been studied using the mouse and rat as model systems.

AFP is believed to be synthesized during fetal development by endodermally derived cells, e.g. visceral endoderm layer of the yolk sac and parenchymal liver cells(4). The synthesis of AFP and its regulation have been studied most intensively in the liver. The level of serum AFP is dependent on the number of hepatocytes which synthesize AFP (4). Fetal hepatocytes that synthesize AFP also synthesize albumin, transferrin and other serum proteins (75). Only albumin and transferrin are synthesized in the adult liver, suggesting that AFP expression is selectively inhibited during ontogeny.

Several studies have suggested that the inhibition of AFP synthesis is controlled by extrinsic factors. A protein factor isolated from adult tissues, when injected into newborn mice, reduced the level of circulating AFP (218). Similarly, glucocorticoids reduce AFP levels in newborn rats (23). Whatever the physiological inhibitory mechanism, it is very efficient, reducing the serum levels by 10^{-5} in most species (184), however in some strains of mice, the serum level is very high indicating a possible mutation in the inhibitory mechanism. Genetic experiments suggest that a single gene is responsible for the control of AFP synthesis, and that in the normal adult it regulates and maintains the low level of synthesis normally seen (151).

Several model systems have been developed to study aberrant re-expression of AFP during the adult stage. Liver injury produces increases in serum AFP. Liver injury induced by partial hepatectomy or carbon tetrachloride has been shown to increase AFP synthesis in rats and mice (58, 184). The cells that synthesize AFP appear to be normal hepatocytes (59). As in the fetal liver, the cells of the damaged liver which synthesize AFP also produce albumin and transferrin (75). The synthesis of AFP in these cases is associated with increased cell division, (59, 163, 184), and AFP is detected during G_1 and S phases of the cell cycle. However, differences exist between induction by hepatectomy and hepatotoxin treatment. The experiments of Watanabe *et al.* suggest that toxin-induced AFP synthesis is related to cell replication while hepatectomy induction is not (229). The conclusion derived from these experiments is that AFP synthesis can be induced in normal hepatocytes, indicating a retrodifferentiation step by the cell to a more embryonic state.

AFP synthesis is induced in rats by certain carcinogens (227). During chemically induced hepatocarcinogenesis there is an initial small, transient increase in AFP serum concentration which is followed later by a large increase (87). Morphological examination of the liver during this process correlates the early rise of AFP synthesis with the appearance of new cell types. It has been proposed that they are precursors of adult hepatocytes, i.e. "oval" cells and the "transitional" cells (48, 99). The secondary rise in AFP is due to the appearance of tumor cells (88).

The synthesis of AFP by different hepatoma cell lines varies from zero to very high levels (20, 21). In these studies the level of AFP synthesis correlated with the rate of growth, state of differentiation and ploidy. An exception is the Morris 7777 tumor line which produces large quantities of AFP but is near diploid except for a chromosome abnormality on chromosome 7 (243). These various tumors would also provide model systems for the study of control of AFP expression.

The expression of AFP in these various systems has been evaluated but the mechanisms that control the changes in expression are poorly understood. Initial investigations into the regulation of AFP and albumin synthesis during fetal development of the mouse showed that the level of AFP synthesis is directly related to the amount of AFP mRNA present (108, 109, 140, 204, 205). Similar results have been obtained in studies of adult liver and hepatoma (40, 96, 177), and of yolk sac and yolk sac tumor (104). Dexamethasone reduces the AFP levels in newborn rats (23) and this has now been shown to be due to a decrease in the level of AFP mRNA (42). These studies suggest that AFP synthesis is controlled at the transcriptional rather than the translational level.

The structure of the AFP gene is now under investigation. The gene has been shown to be 8 - 10 times larger than the coding sequences and contains at least 11 introns (77, 137). During development and neoplastic transformation, the gene does not undergo gross rearrangements, suggesting that transcription may be the key control point (178). This possibility has also been suggested by Innis and Miller who showed that the level of AFP mRNA correlated with the rate of transcription of the AFP gene (97). The control of transcription is a complex problem and for control of AFP expression, many factors remain to be elucidated.

C. Mechanisms of Control of Gene Expression

During development, the patterns of protein synthesis change in a temporal fashion (7), presumably due to differential gene expression, eventually leading to a differentiated state where certain specific proteins are synthesized in relatively large amounts (110, 131). The processes through which information encoded in the DNA is finally expressed as protein are quite complex and include three stages: transcription of the gene, processing of the RNA and translation of the mRNA.

Control of gene expression during differentiation is believed to be mainly at the transcriptional level (152). Transcriptional control may involve structural changes in DNA such as deletions, duplication, rearrangement and methylation. For instance, gross rearrangement of the DNA has been shown to be involved during the activation of the immunoglobulin genes (211). The DNA of active genes is often hypomethylated at cytosines in comparison to that of inactive genes (113, 129, 202, 222). This modification is believed to take place early in development during the determination of the cell lineages (222).

Proteins associated with DNA are believed to contribute to the structural integrity and differential expression of the genome. These proteins can be divided into two main groups, histones and non-histones. Five types of histones are known to exist, playing primarily a structural role. Four of the histones are involved in the formation of nucleosomes (136). The nucleosomes are found in both the active and inactive regions of the chromatin. The histones can be modified in several ways which may have functional significance. For instance, the active genes are enriched with acetylated histones (11, 98, 132). The non-histone proteins, on the other hand, are believed to be directly involved in the control of transcription (152). They are a heterogeneous group containing enzymes, regulatory and structural proteins. They show tissue and developmental specificity (37, 207). In *in vitro* transcription assays, these proteins can control the expression of specific genes (39, 197). The regulatory proteins are thought to work in both a positive fashion by promoting the binding of RNA polymerase to specific initiation sequences on the DNA and in a negative fashion by inhibiting this process (24, 158, 226). The configuration of active genes is more "open" than that of inactive ones as measured by DNase sensitivity (230). This sensitivity is due to two non-histone proteins, HMG 14 and 17 which are bound to the nucleosomes of active chromatin (179, 231).

The initial RNA transcript is much larger than the final form of RNA found in the cytoplasm. The coding sequence of the final mRNA is interspersed with non-coding sequences called introns (19, 69, 180, 209). As the RNA is synthesized, it becomes associated with proteins to form a ribonucleoprotein particle (RNP) (159, 164). Soon after the initiation of the RNA molecule, the 7 ^{Me}G cap is added,and when transcription is terminated, the poly (A) tail is attached (44, 143, 245). DNA sequences have been identified which are implicated in these modifications (24, 55, 198). These modifications are believed to be involved in mRNA translation and stability. Furthermore, the intron regions are removed by enzymes most likely present in the RNP and possibly with the aid of small nuclear RNA's (17, 54, 119, 168, 70). These modifying reactions are believed to be important in gene regulation in that alternative sites of poly (A) addition or RNA splicing could lead to differential gene expression (44, 105). A possible example of the former mechanism is shown by the IgM system (54).

The mature mRNA is transported to the cytoplasm in the form of a RNP particle (164). In certain systems, mRNA's are stored in the cytoplasm in these complexes until they are required at a specific point in development (78, 196). The proteins bound to the RNA in the cytoplasm may also play a role in stabilizing mRNA's. The differential stability of mRNA's would allow for the accumulation of specific mRNA (126, 169). The stability of mRNA's has been shown to be variable in hormone and chemically induced systems, suggesting that factors other than RNA structure, possibly bound proteins, are involved in mRNA stability (94, 127, 169).

The rate of translation is dependent on many factors. The cap structure appears to be important for the efficient translation of mRNA (65, 188). Many protein factors are involved in the initiation and elongation during translation (47, 183). The affinity of different mRNA's for these factors may lead to differences in the initiation and elongation of a protein (103, 125). The sequence of the RNA is probably involved in these interactions and therefore could indirectly control the rate of translation. The initiation and termination sequences on the RNA are important to produce the proper, functional protein (85, 112). Translation does not appear to be a mechanism of qualitative gene control but may be important in quantitative control.

Proteins which are destined for secretion or membrane insertion have a signal sequence encoded in the RNA. This signal sequence allows the protein to move across the membrane of the E.R. (145, 199). Membrane proteins become integrated during this process, while proteins destined for secretion move entirely into the lumen of the E.R. from where they are secreted.

CHAPTER II

MATERIALS AND METHODS

A. Yolk Sac

(1) Isolation

Swiss Webster mice were allowed to mate overnight and the presence of a vaginal plug the morning after mating indicated day 0.5 of pregnancy. The yolk sacs were removed under aseptic conditions at various days of gestation. The yolk sacs were rinsed several times in sterile PBS (15 mM sodium phosphate, 150 mM NaCl, pH 7.2) and the amnion was removed.

(2) Culture Conditions

The yolk sac explants were incubated under constant agitation in Eagle's Minimal Essential Medium containing non-essential amino acids, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C. Details of any modifications to the medium and addition of radioactive isotopes are given in the appropriate sections.

B. Assay of Macromolecules in Yolk Sac Explants

(1) DNA

(a) Determination of total DNA

Yolk sacs that had been cultured in Minimal Essential Medium were lysed by three cycles of freeze and thaw in the presence of 1% sodium deoxycholate and Triton X-100. The crude lysates were centrifuged at 15,000 xg for 25 min and DNA was extracted from both the pellets and supernatants. Pellets were dissolved in 0.5 N NaOH at 80°C for 20 min, neutralized with an equal volume of 0.5 N HCl and precipitated with 10% TCA. The precipitates were extracted with 95% ethanol at 65°C for 5 min, pelleted at 8,000 xg for 10 min and then hydrolyzed in 0.3 N PCA at 70°C for 30 min. After cooling on ice, the samples were centrifuged at 8,000 xg for 10 min, and the supernatants were analyzed for deoxyribose content by the diphenylamine reaction (33). To assay DNA in lysate supernatants, the samples were precipitated with 10% TCA and the precipitates were treated with 0.5 N NaOH, neutralized with 0.5 N HCl and then precipitated with 10% TCA. These precipitates were extracted with hot ethanol and the DNA was pelleted at 8,000 xg for 10 min. The DNA was hydrolyzed and analyzed as described above.

(b) DNA synthesis

Each yolk sac explant was minced with scissors and placed in a sterile 16 X 150 mm test tube along with 0.3 ml of medium supplemented with 10% fetal calf serum. Each tube was gassed with 5% CO_2 - 95% air, sealed, and incubated for 30 min. Reactions were initiated by addition of 2 μCi of [6-^{3}H] thymidine (2 Ci/mmole, Amersham) and terminated at various times thereafter by the addition of 2 ml cold 5% PCA. The resulting precipitates were collected by centrifugation, washed three times with 2.0 ml cold 5% PCA and dissolved in 1.5 ml NCS (Amersham) at 37°C overnight. Samples were neutralized with two drops of glacial acetic acid and the radioactivity of the total sample was measured in 10 ml of scintillation cocktail (4 g of 2,5-diphenyloxazole and 50 mg of 1,4 Bis-(2-(5-phenyloxazolyl)) benzene per liter of toluene).

(2) RNA

(a) Total RNA

The amount of RNA was determined by measuring the absorbance at 260 nm. 1 mg/ml of RNA has an A_{260} of 20.

(b) RNA synthesis

The synthesis of total RNA in yolk sac explants was determined as described for total DNA synthesis except that 2 μCi of [5-^{3}H]uridine (25 Ci/mmole, Amersham) was used instead of [^{3}H] thymidine.

(c) Pulse and chase experiments

To determine the conditions for the pulse and chase experiments, yolk sac explants from day 11.5, 15.5 and 17.5 were cultured in medium described in section A (2) containing 15 mM Hepes and 0.25 mCi/ml of [5,6-^{3}H] uridine (50 Ci/mmole, Amersham). At various times after initiation of the culture, three yolk sacs were removed from the culture flask, homogenized in 3.0 ml of 10 mM Tris pH 7.4, 5 mM EDTA with a motor driven teflon pestle-glass homogenizer and then frozen. In chase experiments, the radioactive medium was replaced after 2 hr of incubation with 1.5 volumes (day 11.5), 2.0 volumes (day 17.5) or 3 volumes (day 15.5) of medium containing 5 mM uridine, 5 mM cytidine and 5 mM glucosamine. Yolk sacs were removed at various times, homogenized as described above, and frozen. Samples were later thawed and analyzed in triplicate for radioactive RNA by cold TCA precipitation as described below in section B (2)(d).

For the isolation of RNA during the pulse and chase, yolk sac explants from day 11.5, 15.5 and 17.5 were cultured in medium described above at 2-3 yolk sacs/ml. After incubation for 4 hr, some of the yolk sacs were removed from the medium and frozen on dry ice. The remainder were washed once with pre-warmed chase medium, containing 5 mM uridine, 5 mM cytidine and 5 mM glucosamine and incubated in 3 volumes of the same medium. After the initiation of the chase, yolk sacs were removed at various times and stored frozen. The chase was terminated within 30 hr as the viability of the yolk sacs in culture for longer periods is unknown. RNA was extracted from the yolk sacs and the radioactivity was determined as described below.

(d) Preparation of total radioactive RNA

Yolk sac explants were incubated in medium described in section A (2) containing 15 mM Hepes and 0.25 mCi/ml of [5,6-^{3}H] uridine (50 Ci/mmole, Amersham) at 2-3 yolk sacs/ml for 4 hr, and then they were quickly frozen. The frozen yolk sacs were homogenized at room temperature in the presence of 5 volumes of SEN (0.5%SDS, 25 mM EDTA, 75 mM NaCl pH 8.0) and 5 volumes of SEN saturated phenol-1% 8-quinolinol using 15 strokes of a loose dounce homogenizer. The homogenate was chilled on ice for 30 min and centrifuged at 16,000 xg for 10 min. The aqueous phase and the proteins at the interphase were re-extracted with 2.5 volumes of phenol and 2.5 volumes of chloroform-isoamyl alcohol (24:1) by vortexing for 5 min. Phase separation was repeated as above, the aqueous layer was made 0.2 M with NaCl and 2 volumes of cold ethanol were added. The nucleic acids were precipitated overnight at -20°C and the precipitate was collected by centrifugation at 16,000 xg for

20 min. The pellet was dissolved in 20 mM EDTA by heating at 70°C for 1 to 3 min. Three volumes of 4.0 M sodium acetate, pH 6.0, were added and the mixture was kept on ice for 30 min. The precipitate that formed was collected by centrifugation at 16,000 xg for 20 min. The acetate extraction was repeated twice. The final pellet was dissolved in SEN solution, Proteinase K was added to 50 µg/ml and the solution was incubated at 37°C for 30 min. RNA was extracted with phenol-chloroform and precipitated with ethanol as described above. The RNA was collected by centrifugation at 16,000 xg for 20 min, dried and dissolved in sterile water. The RNA was analyzed spectrophotometrically and the A_{260}/A_{280} was usually greater than 1.8 and the A_{260}/A_{230} was greater than 2.25. The radioactivity was determined by precipitating a small sample of the RNA solution along with 20 µl of BSA (5 mg/ml) with cold 10% TCA. The precipitate was collected on Whatman GF/A filters and washed with 10% TCA and radioactivity on the filter was determined.

(e) Preparation of poly (A)$^+$ RNA

Poly (A)$^+$ RNA was prepared as described by Aviv and Leder (16). Oligo d(T) cellulose Type 3 (Collaborative Research) was suspended in binding buffer (0.5 M NaCl, 1 mM EDTA, 10 mM Tris pH 7.4, 0.5% SDS) and poured into a sterile 1 ml syringe plugged with sterile glass wool. The column was washed with binding buffer, then with elution buffer (10 mM Tris pH 7.4, 1 mM EDTA and 0.05% SDS) and finally equilibrated with binding buffer. The sample in 1.1 mM EDTA, 11.1 mM Tris pH 7.4 and 0.58% SDS, was heated at 45°C for 5 min and then NaCl was added to 0.5 M. The sample was applied to the column at a flow rate of 0.5 ml/min and the unbound fraction was collected. The column was washed with an equal volume of binding buffer. The two unbound fractions were pooled, reapplied to the column and the unbound fraction was recollected. The column was washed with binding buffer and the wash fraction was collected. The bound fraction was eluted with a small volume (less than 1 ml) of elution buffer. The radioactivity in the different fractions was determined by the cold TCA method described in section B (2)(d).

(f) Assay of AFP mRNA by filter hybridization

The plasmid pAF-7 consists of 5200 base pairs of DNA, of which 900 were derived from cDNA complementary to 42% of AFP mRNA (gift from S. T. Law, Baylor College of Medicine) (118). The plasmid was linearized by treatment with the restriction enzyme Eco R1. Reaction mixtures contained 100 mM Tris pH 7.2, 5 mM $MgCl_2$, 50 mM NaCl, 2 mM 2-mercaptoethanol and 1-2 units of Eco R1 (BRL Lyphozyme) per μg plasmid DNA and were incubated at 37°C for 5 hr. SDS and Proteinase K were added to final concentrations of 0.5% and 50 μg/ml, respectively and incubated for 40 min at 37°C. The DNA was extracted once with phenol-chloroform (section B (2)(d)). The aqueous phase was recovered and stored at 4°C.

The linearized DNA preparation (250-300 ug/ml) was denatured by a 10 fold dilution with 0.4 M NaOH followed by incubation at room temperature for 10 min and 4°C for 5 min and then diluted with an equal volume of 2.0 M ammonium acetate pH 7.0. Approximately 100 μg of DNA was applied to nitrocellulose filters (Schleicher and Schuell, BA 85, 0.45 μm and 25 mm diam.) which had been washed with 5.0 ml of 1.0 M ammonium acetate pH 7.0. The filters were washed with 5.0 ml of 1.0 M ammonium acetate, pH 7.0, air dried for 1 hr and baked at 75°C for 4 hr. Filters were gently shaken for 3 hr at room temperature in Denhardt's solution (0.02% bovine serum albumin, 0.02% Ficoll type 400, 0.02% polyvinyl pyrrolidone 360, 4x SSC (600 mM NaCl, 60 mM sodium citrate pH 7.0))(49). They were blotted and baked at 75°C for 2 hr. Small 7 mm diameter filters were punched from each filter. Each small disc contained about 10 μg of plasmid DNA. Control filters were prepared as described above except that no DNA or sonicated denatured chicken DNA was pumped through the filter. Hybridization reactions contained 100 μl of SEH buffer (600 mM NaCl, 3 mM EDTA, 10 mM Hepes pH 7.4), 10 μg poly (A) (P/L Biochemicals), 0.1% SDS, [^{3}H] RNA (Section 2 (c)), 1 plasmid containing filter and 1 control filter. The RNA was heated at 68°C for 3 min before adding it to the hybridization solution. The hybridization solution was incubated at 66°C for 18 hr. The buffer was removed and the filters were washed extensively and treated with RNase A to remove any non-specifically bound RNA (214). The filters were then dried and counted. Net cpm bound was calculated by subtracting the radioactivity on the control from that on the plasmid containing filter.

(3) Proteins

(a) Determination of total protein

The BioRad Protein Assay Kit was used to quantitate the amount of protein present in the yolk sac lysates (28). Four μl of lysate was mixed with 800 μl of PBS pH 7.2, 200 μl of dye reagent concentrate was added and the sample was mixed by gentle vortexing. After 20 to 30 minutes at room temperature, the absorbance at A_{595} of the samples was determined. Bovine gamma globulin was used as a calibration standard. Sample values were corrected for background reaction due to detergent alone.

(b) Assay of protein synthesis in yolk sac explants

Yolk sac explants were incubated in medium containing 15 mM Hepes,pH 7.2 for 30 min. The incubation medium was then replaced with pre-warmed L-leucine-free medium containing 80 μCi/ml [^{3}H] leucine (48 Ci/mmole, Amersham) or L-methionine-free medium containing 50 μCi/ml [^{35}S] methionine (935 Ci/mmole, New England Nuclear) and the incubation continued at 37°C. The labelling time varied with each experiment. To stop the isotope incorporation and lyse the yolk sac cells, the cultures were cooled on ice and sodium deoxycholate, Triton X-100 and L-leucine or L-methionine were added to 0.5%, 0.5% and 1 mM, respectively. The samples were frozen and thawed three times, the detergent concentrations were increased to 1% each and the freeze and thaw procedure was repeated three more times. The protease inhibitor PMSF was added to a final concentration of 1 mM for the lysis of methionine-labelled yolk sacs. The lysates were clarified at 100,000 xg for 60 min at 4°C and frozen.

To measure the incorporation of radioactive amino acids into total protein, 2.0 ml of cold 10% TCA was added to 10 - 25 μl of lysate to which 25 - 30 μl of 5 mg/ml BSA had been added as carrier. The sample was placed in a boiling water bath for 15 min and then cooled in ice. The precipitate was collected on Whatman glass fiber filters (GF/A), dried and dissolved in 0.5 ml NCS at 60°C for 1 hr. The sample was neutralized with 2 drops of glacial acetic acid, scintillation fluid was added and the radioactivity was determined.

(c) Assay of protein synthesis and secretion by yolk sac explants

Yolk sac explants were cultured as described in the previous section. The yolk sacs were removed from the medium, rinsed twice in PBS and lysed in PBS by the detergent-freeze and thaw method. This is referred to as the cellular fraction. The medium was made up to 1 mM in PMSF and 1 mM in methionine and was stored frozen. Later the medium was thawed and centrifuged in an Eppendorf microfuge for 15 min at 4°C. The supernatant was stored frozen and is referred to as the secreted fraction. These two fractions, i.e. the cellular and secreted fractions, were analyzed for total radioactive protein by the hot TCA precipitation method and by two dimensional gel electrophoresis.

(d) Electrophoretic analysis

(i) SDS PAGE

Electrophoretic analysis was performed on slab gels (1.5 mm thick) according to Laemmli (115). For the analysis of immunoprecipitates, a 10% acrylamide separating gel (10 cm) was used with a 3% stacking gel (2.0 cm). For analysis of total protein in lysates, an 8 to 16% linear polyacrylamide gradient was used with a 3% stacking gel. Samples containing an equal amount of radioactivity were adjusted to 3% SDS, 10% glycerol, 5% 2-mercaptoethanol and 62.5 mM Tris, pH 6.8 (SDS sample buffer) and heated at 90°C for 10 min. Bromophenol blue (0.2%, 3 µl per slot) was used as a tracking dye.

Electrophoresis was carried out at 10 mA per gel until the dye passed through the stacking gel, then the current was increased to 20 mA per gel. Electrophoresis was stopped when the tracking dye reached the bottom of the gel. The gel was stained with 0.05% Coomassie Brilliant Blue (G250) in methanol, water and glacial acetic acid (5:5:1) for at least 2 hr and was destained in 7.5% acetic acid, 5% methanol in water. Radioactive protein bands were located by fluorography using Kodak XR-1 X-ray film according to Bonner and Laskey (27, 117).

(ii) Two dimensional gel electrophoresis

Two dimensional gel electrophoresis was carried out essentially according to O'Farrell (150). Samples of cellular and secreted fractions were made 0.45% in SDS, 1.5% in glycerol, 0.75% in 2-mercaptoethanol, 10 mM in Tris pH 6.8 and heated at 70°C for 20 min. After cooling, urea, Nonidet P-40, 2-mercaptoethanol and ampholines (pH 4-6: pH 6-8: pH 3-10, 2:2:1, Biorad) were added to the samples to give final concentrations of 9 M, 2%, 5% and 2% respectively. These samples were layered onto gels containing 9.2 M urea, 2% Nonidet p-40, 2% ampholines and 4% acrylamide, which were cast in 3 mm diameter tubes. The gels were run at 350 volts for 19 hr, and then removed from the glass tubes and frozen. This method of isoelectric focussing produced a pH gradient from 4 to 8. The gels were later thawed, equilibrated with SDS sample buffer (section B.3.d(i)) for 1 hr, then layered onto an 8 to 16% linear gradient slab gel. One percent agarose was used to hold the isoelectric focussing gel in place during electrophoresis. Electrophoresis and treatment of the gel are described under SDS PAGE.

(iii) Alkaline gel electrophoresis

A 30 cm long slab gel was formed with a 2 cm stacking gel using solutions described by Davis (46). The separating gel was 7% polyacrylamide and was at pH 8.9. Bromophenol blue and 40% sucrose were added to the sample before the sample was layered onto the gel. The samples were run through the stacking gel at 12 mA/gel and then at 20 mA/gel through the separating layer. Gels were stained with Coomassie Brilliant Blue and prepared for fluorography as described above.

(e) Immunoprecipitation analysis

(i) Antibody preparation

For the preparation of AFP antibody, partially purified mouse AFP (2 mg/ml) was mixed with an equal volume of Complete Freunds Adjuvant and 0.5 to 1.0 mg of protein was injected into each rabbit subscapularly (140). Four similar weekly injections were repeated using Incomplete Freunds Adjuvant. One week after the last injection, blood was collected by cutting the lateral ear vein. The antiserum was isolated and absorbed three times with adult mouse serum and the IgG fraction was precipitated with 18% Na_2SO_4. The IgG fraction was taken up in 1/10 the original volume of 10 mM Tris-saline and dialyzed against Tris saline overnight. The antibody preparations were stored lyophilized. During the purification, the specificity and activity were checked using the Ouchterlony double diffusion method. The preparation was rehydrated to a protein concentration of 90 mg/ml.

Freeze-dried goat anti-mouse transferrin IgG fraction was purchased from Cappel Laboratories Inc. The antibody preparation was rehydrated with 2.0 ml water. The equivalence of the transferrin antibody was 40 μl of antibody to 2.5 μl of mouse serum and 40 μl of antibody to 40 μl of amniotic fluid.

(ii) Immunoprecipitation assay

Yolk sac lysates containing radioactive proteins were used to find immunoprecipitation conditions where the appropriate antibodies would quantitatively precipitate AFP and transferrin. In these experiments increasing amounts of antibodies were added to equal volumes of lysate containing carrier protein (AFP or transferrin). The reaction mixtures were incubated at 28°C for 1 hr, then at 4°C overnight. The precipitate was centrifuged at 10,000 xg for 15 min through a 0.9 M sucrose cushion containing 1% sodium deoxycholate, 1% Triton X-100, and 1 mM leucine or methionine, in PBS, pH 7.2. The pellets were resuspended in PBS containing 1% sodium deoxycholate, 1% Triton X-100 and 1 mM leucine or methionine, and washed two more times through sucrose cushions. The final pellet was dissolved in either 50-100 μl of SDS sample buffer (see above) by heating at 90°C for 15 min, or in NCS. The radioactivity in the precipitate was determined. For the remainder of the experiments, antibody was always in excess, allowing for the quantitative precipitation of AFP and transferrin.

(f) Assay of glycosylation

Yolk sac explants from day 15.5 were incubated in medium (Section A(2)) containing 15 mM Hepes at 2 yolk sacs/ml for 30 min.

Two hundred µCi of D-[1,6-^{3}H(N)] glucosamine HCl (39.6 Ci/mmole, New England Nuclear) was added and the incubation continued for 1.5 hr. The yolk sacs were removed from the culture medium and lysed as previously described (section B.3(b). Samples were analyzed by SDS gel electrophoresis and two dimensional gel electrophoresis. The labelled glycoproteins were then visualized by fluorography.

(4) Inhibitor experiments

(a) Tunicamycin--inhibitor of glycosylation

Five yolk sacs were incubated in 2.5 ml of medium in the presence of tunicamycin (Lilly Research Laboratories) at a concentration of 2 µg/ml. For the 2 hr samples, yolk sacs were incubated for 30 min at 37°C in medium described in section A.2, followed by a change of medium to either methionine-free medium containing 50 µCi/ml of [^{35}S] methionine or medium containing 80 µCi/ml of [^{3}H] glucosamine. The incubation was continued for 1.5 hr. For the 6 hr samples, the yolk sacs were incubated for 4.5 hr, then switched to isotope containing medium for 1.5 hr. The yolk sacs were separated from the medium and treated as described above to yield cellular and secreted protein fractions. These were analyzed for total protein, incorporation of [^{35}S] methionine into total protein, and AFP and also incorporation of [^{3}H] glucosamine into total glycoprotein and AFP. [^{35}S] labelled protein fractions were analyzed by SDS and two dimensional electrophoresis.

(b) α-Amanitin--inhibitor of RNA and protein synthesis

Day 15.5 yolk sacs were cultured essentially as described above in the presence or absence of 10 µg/ml of α-amanitin (Boehringer Mannheim). Cultures were incubated for 1.5, 7.5 and 25.5 hr. [^{35}S] methionine was present in the culture medium for the last hr of incubation. The yolk sacs were removed from the culture medium at the end of the incubation and lysed as described above. The lysates were analyzed for protein content, total protein synthesis and AFP synthesis. Proteins were analyzed by two dimensional gel electrophoresis.

C. Nuclei

(1) Isolation

This method of isolating nuclei from yolk sacs was a combination of several procedures (34, 213). The yolk sacs were homogenized in 5 volumes of 0.32 M sucrose in TMK buffer, (10 mM Tris pH 7.9, 5 mM magnesium acetate, 25 mM potassium chloride, 1 mM 2-mercaptoethanol) supplemented with 0.1% Triton X-100, using a motor-driven teflon pestle-glass homogenizer. The homogenate was filtered through 4-6 layers of cheesecloth and the filtrate was centrifuged at 600 xg for 10 min. The pellet was resuspended in 5 volumes of the above buffer using a sterile glass rod and then sedimented through 0.88 M sucrose in TMK at 600 xg for 20 min. The pellet was resuspended in 5 volumes of 2.1 M sucrose in TMK and the nuclei were pelleted at 20,000 xg for 25 min. The pellet was taken up in 1 volume of TGDEM (10 mM Tris pH 7.9, 25% glycerol, 10 mM dithiothreitol, 50 μM EDTA, 5 mM magnesium acetate) and centrifuged at 600 xg for 10 min. The final pellet was resuspended in a small volume of TGDEM and frozen at -70°C.

To assay DNA content, nuclei were precipitated by cold 10% TCA. The precipitate was collected by centrifugation in a clinical centrifuge for 5 min. The pellet was resuspended in 10% PCA and heated at 85-90°C for 30 min. The sample was then cooled on ice, centrifuged and the supernatant was analyzed for deoxyribonucleotides by the diphenylamine reaction (33).

(2) RNA Synthesis

The nuclei were thawed slowly and incubated at 25°C in 10% glycerol, 20 μM EDTA, 4 mM dithiothreitol, 50 mM Hepes pH 8.0, 1 mM manganous chloride, 5 mM magnesium acetate, 150 mM potassium chloride, 1.2 mM ATP, 1.0 mM GTP, 1.0 mM CTP and 0.4 mM [^{3}H] UTP (1-3.5 Ci/mmole). The inhibitors, α-amanitin and actinomycin D, were used at 3 μg/ml and 20 μg/ml, respectively. The nucleic acids in 10-15 μl samples of the incubation mixture were co-precipitated with carrier BSA (30 μl of 5 mg/ml solution) using cold 10% TCA - 50 mM sodium pyrophosphate. The precipitates were collected on Whatman GF-A filters, washed with cold 10% TCA - 50 mM sodium pyrophosphate, dried and the radioactivity on filters was determined.

CHAPTER III

DEVELOPMENTAL CHANGES IN MACROMOLECULAR SYNTHESIS IN THE YOLK SAC

A. Introduction

Previous studies on the yolk sac have shown that there are changes in structure and function during gestation. The physical characteristics of the yolk sac change several days before birth (83). The microvilli of the endodermal brush border become shorter. The storage of glycogen and lipid, and enzymatic activity change with gestation (153, 195). Recently, changes in the activity of degradative enzymes with development have been detected biochemically in the mouse (190). The transmission of antibodies is more efficient near the end of gestation (29). The synthesis of collagen, which is an integral component of the basement membrane, changes from type IV to type I (5). Sialyltransferase activity increases with development (128). These results indicate that the yolk sac undergoes biochemical changes during gestation and these changes relate to its structure and function.

To further investigate gestational changes in the yolk sac at the biochemical level, the syntheses of the three major classes of macromolecules: DNA, RNA and protein were studied. The experiments were done *in vitro* with the assumption that short term explant culture would reflect *in vivo* activity and allow comparison of the different stages of gestation.

B. Results

(1) Growth of the Mouse Yolk Sac During Gestation

During the last half of gestation, the mouse fetus is surrounded by amniotic fluid contained inside a compartment formed by the yolk sac and the placenta (Fig. 3). The fetus at day 11.5 is just beginning to develop unique features and is difficult to see through the yolk sac at this stage of development (Fig. 3A). By days 15.5 and 17.5 (Fig. 3B and C) the fetus has developed many distinctive features, e.g. limbs, head, tail and liver, and is more easily visualized.

The yolk sac does not undergo morphological changes from day 10.5 to 18.5. The predominant feature of the yolk sac is the vitelline vessels, which have formed by day 10.5 and are present during the remainder of gestation. The main change that occurs during yolk sac development is a dramatic increase in size from day 11.5 to day 17.5 in keeping with the rapid growth of the fetus.

To quantitate the growth of the yolk sac during gestation, the wet weight, protein content and DNA content were determined (Fig. 4). The wet weight increased 2.5 fold from day 13.5 to day 15.5, after which the rate of growth seemed to decrease (Fig. 4A). The wet weight at earlier days of gestation was difficult to determine accurately. The protein content of yolk sac lysates (Fig. 4B) increased linearly up to day 17.5, after which a slight decrease was observed. The amount of DNA per yolk sac also increased during gestation (Fig. 4C); however the rate of increase was not constant. There appeared to be only a slight increase in DNA content from day 11.5 to day 13.5, then a large increase up to day 15.5. From day 15.5 to day 17.5, there was, again, only a small increase in DNA content. The increase in both

Figure 3. The mouse conceptus at different stages of gestation

Photographs were taken of the complete mouse conceptus isolated during the last half of gestation. The main components of the conceptus during this period are the fetus, yolk sac, and placenta.

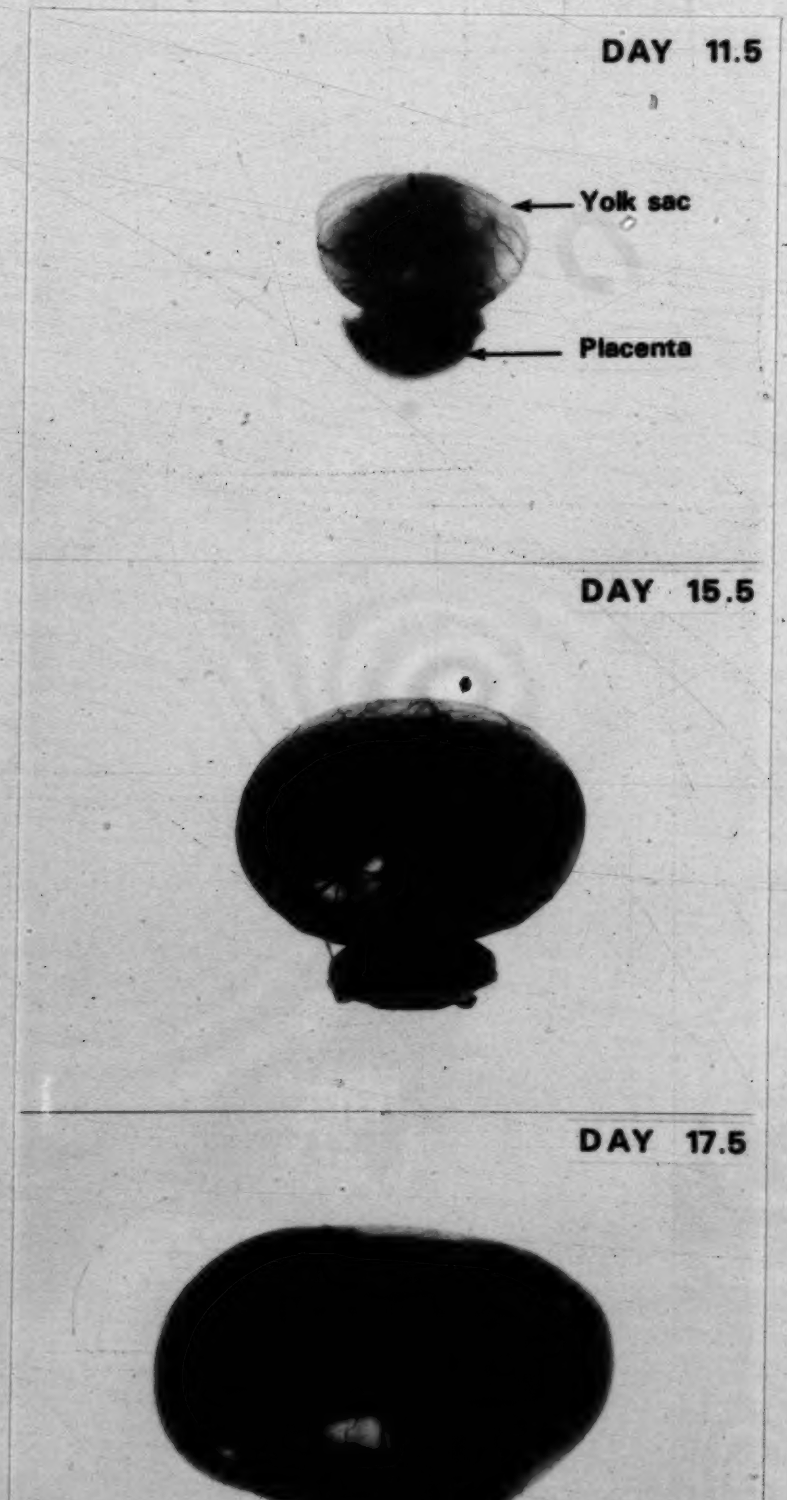
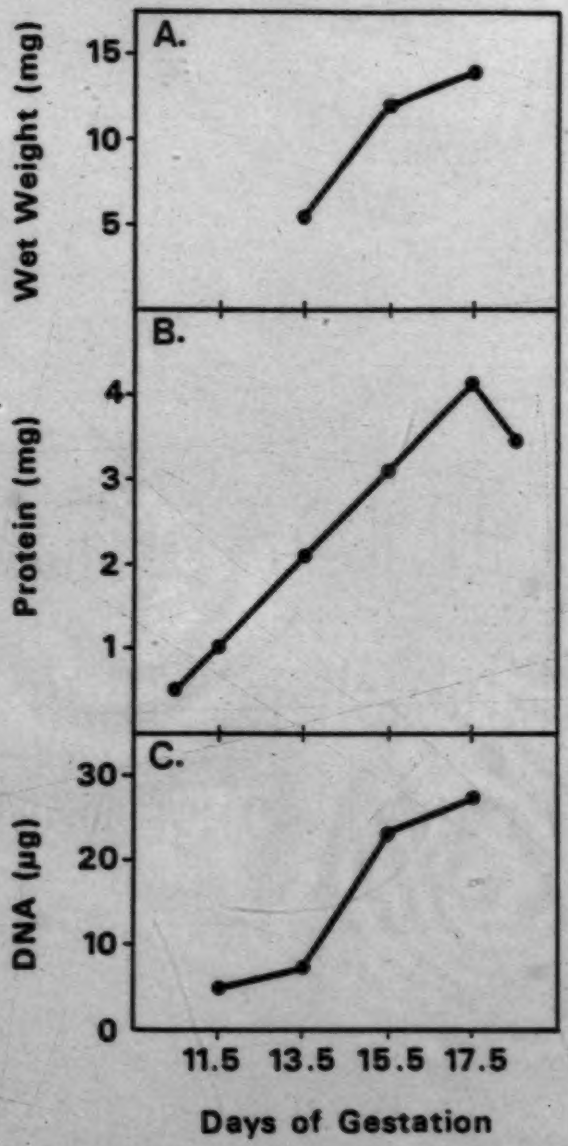

DAY 11.5
Yolk sac
Placenta
DAY 15.5
DAY 17.5
1 cm.

Figure 4. Wet weight, protein and DNA content of mouse yolk sac during gestation. A. Yolk sacs from different stages of gestation were isolated and rinsed in PBS. Excess buffer was removed and the yolk sacs were weighed. Each point is the average of 5 yolk sacs and had a range of about 20%. B. Cultures of 5 yolk sacs were lysed, centrifuged to remove tissue debris and the supernatants were analyzed for protein using the BioRad Protein Assay Kit. Each point is the average of 5 yolk sacs and had a range of 15%. C. DNA was extracted from yolk sac lysates and quantitated by the diphenylamine reaction using calf thymus DNA as a standard. Each point is the average of at least 5 yolk sacs and had a range of approximately 20%.


A.
Wet Weight (mg)
15
10
5
B.
Protein (mg)
4
3
2
1
C.
DNA (µg)
30
20
10
0
11.5
13.5
15.5
17.5
Days of Gestation

protein and DNA content indicated that the growth of the yolk sac is due mainly to an increase in cell number.

(2) Nucleic Acid Synthesis During Gestation

(a) DNA synthesis by yolk sac explants

The kinetics of [^{3}H] thymidine incorporation into DNA by yolk sac explants at days 11.5, 13.5, 15.5 and 17.5 were studied (Fig. 5A). At the early stages, isotope incorporation was linear and quite high, while at the later stages it was very low. In fact, by day 17.5, the incorporation of [^{3}H] thymidine was essentially zero.

The incorporation of [^{3}H] thymidine by yolk sacs during a 3 hr incubation was plotted as a function of time of gestation (Fig. 5B). These results showed a reduction in DNA synthesis of 93% from day 11.5 to 15.5 and then of essentially 100% by day 17.5. When DNA synthesis was expressed as [^{3}H] thymidine incorporation per yolk sac rather than per µg DNA slightly different results were obtained. When expressed this way, DNA synthesis in day 11.5 and 13.5 yolk sacs was the same and DNA synthesis in day 15.5 yolk sacs was only 27% of day 11.5 and 13.5 levels. DNA synthesis in day 17.5 yolk sacs was still very low.

(b) RNA synthesis

(i) Explant culture

The yolk sac cells cease to divide during the last stages of gestation. When cells are no longer actively dividing, other cellular functions such as RNA synthesis may also decrease. To check RNA synthesis during gestation, the incorporation of [^{3}H] uridine into RNA in yolk sac explants was determined (Fig. 6A). Incorporation of uridine

Figure 5. DNA synthesis in yolk sac explants at different stages of gestation.

A. Yolk sacs were isolated from different stages of gestation and cultured individually in medium containing [^{3}H] thymidine. At indicated times, duplicate cultures were removed and the amount of [^{3}H] thymidine incorporated into DNA was determined by PCA precipitation. Day 11.5 (X—X), day 13.5 (0—0), day 15.5 (□—□) and day 17.5 (Δ—Δ). B. The incorporation of [^{3}H] thymidine during a 3 hr incubation (part A) is plotted as a function of gestation.

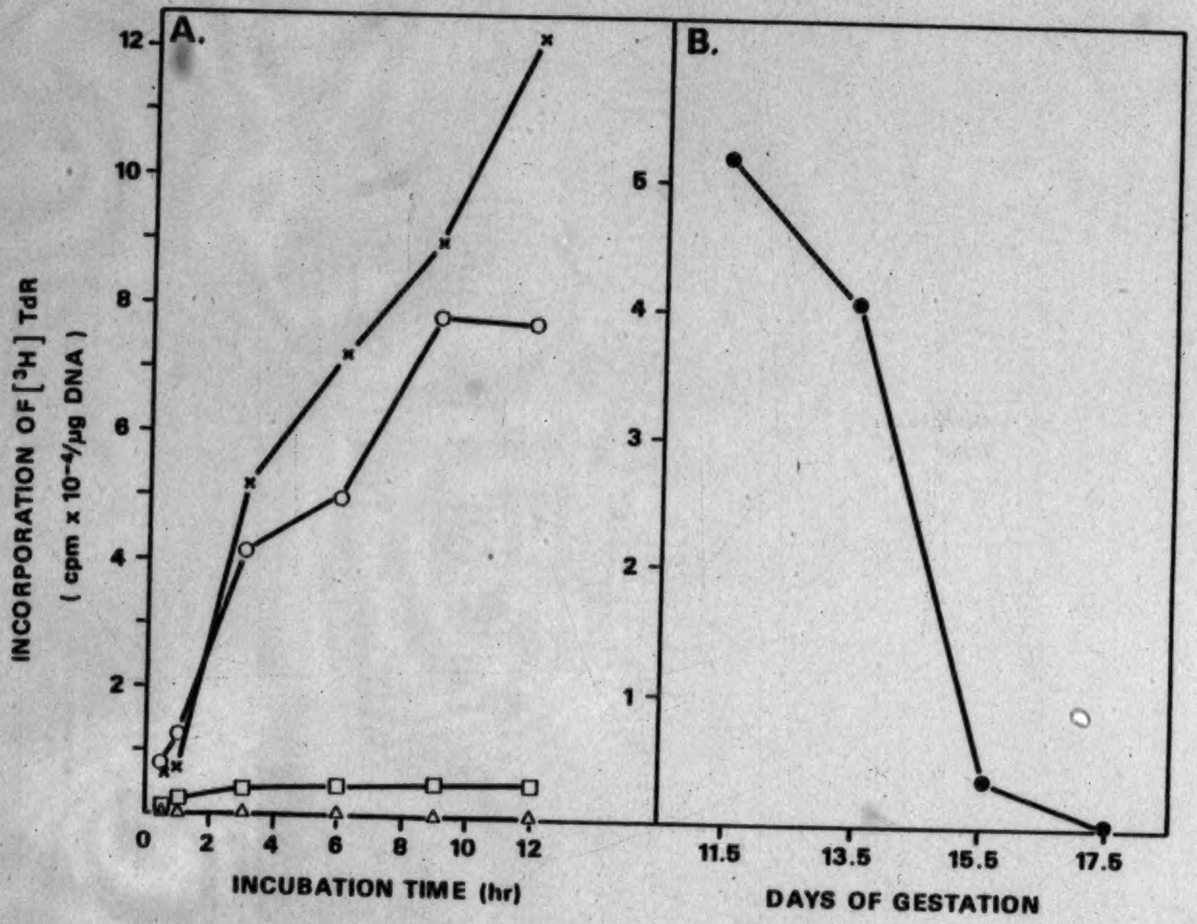
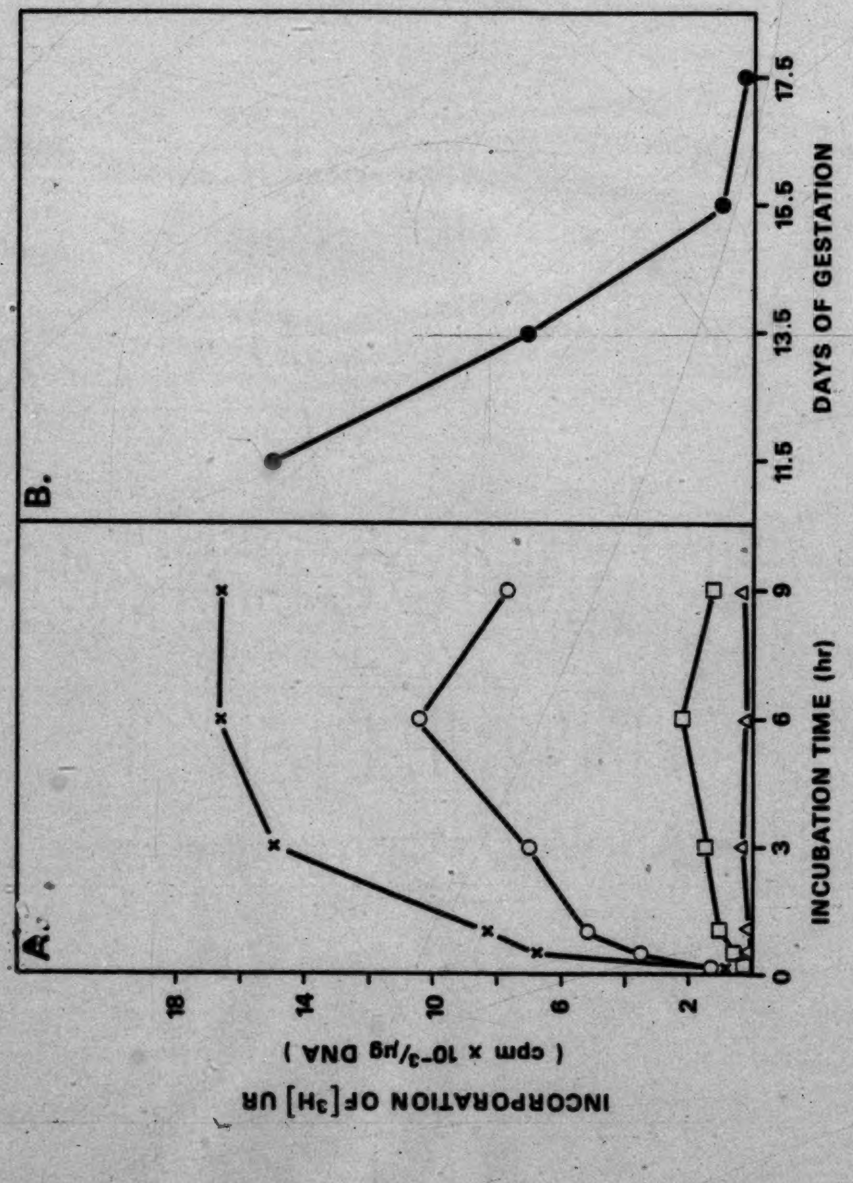

A.
B.
INCORPORATION OF [^{3}H] TdR
(cpm x 10^{-4}/µg DNA)
12
10
8
6
4
2
0
2
4
6
8
10
12
INCUBATION TIME (hr)
5
4
3
2
1
11.5
13.5
15.5
17.5
DAYS OF GESTATION

Figure 6. RNA synthesis in yolk sac explants at different stages of gestation. A. Yolk sacs were isolated from different stages of development and cultured individually in medium containing [^{3}H] uridine. At indicated times, duplicate cultures were removed and the amount of [^{3}H] uridine incorporated into RNA was determined by cold PCA precipitation. Day 11.5 (X—X), day 13.5 (O—O), day 15.5 (□—□), day 17.5 (Δ—Δ). B. The incorporation of [^{3}H] uridine into RNA during a 3 hr incubation (part A) is plotted as a function of gestation.


A.
B.
INCORPORATION OF [3H] UR
(cpm x 10-3/µg DNA)
18
14
10
6
2
0
3
6
9
INCUBATION TIME (hr)
11.5
13.5
15.5
17.5
DAYS OF GESTATION

continued for up to 6 hr of incubation in day 11.5, 13.5 and 15.5 yolk sacs. RNA synthesis in day 17.5 yolk sacs was very low.

The incorporation during a 3 hr incubation was plotted as a function of gestational age (Fig. 6B). The results show that there was a 10 fold decrease between days 11.5 and 15.5, which indicates that RNA synthesis is severely retarded during the later stages of gestation. The pattern of the reduction is very similar to that of DNA synthesis.

There are three main groups of RNA in the cell, ribosomal RNA, transfer RNA and messenger RNA. Poly $(A)^+$ RNA is greatly enriched for mRNA species, while the poly $(A)^-$ RNA consists mostly of rRNA. In preparing the RNA, tRNA was removed by the acetate washes. Total labelled RNA from day 11.5 and 15.5 was separated into poly $(A)^+$ and poly $(A)^-$ RNA and the radioactivity in each fraction was determined (Table 1). For total RNA isolated from both days, there was a 10.1 fold reduction in $[^3H]$ uridine incorporation which was in good agreement with the data in Figure 6. The poly $(A)^+$ fraction had only a 4.2 fold decrease, while poly $(A)^-$ RNA showed a 10.8 fold reduction between the two days. This difference was masked when measuring the total synthesis as mRNA constitutes only 2-3% of the total RNA population. This result suggested that the synthesis of rRNA decreased faster than mRNA synthesis during this period.

(ii) Nuclei

In the previous studies, changes in the incorporation of $[^3H]$ uridine into total cellular RNA were determined. The apparent decrease in RNA synthesis may be due to an increase in the UTP pool with gestation. To test this possibility, nuclei from different days of gestation were isolated and the incorporation of $[^3H]$ UTP into RNA was determined

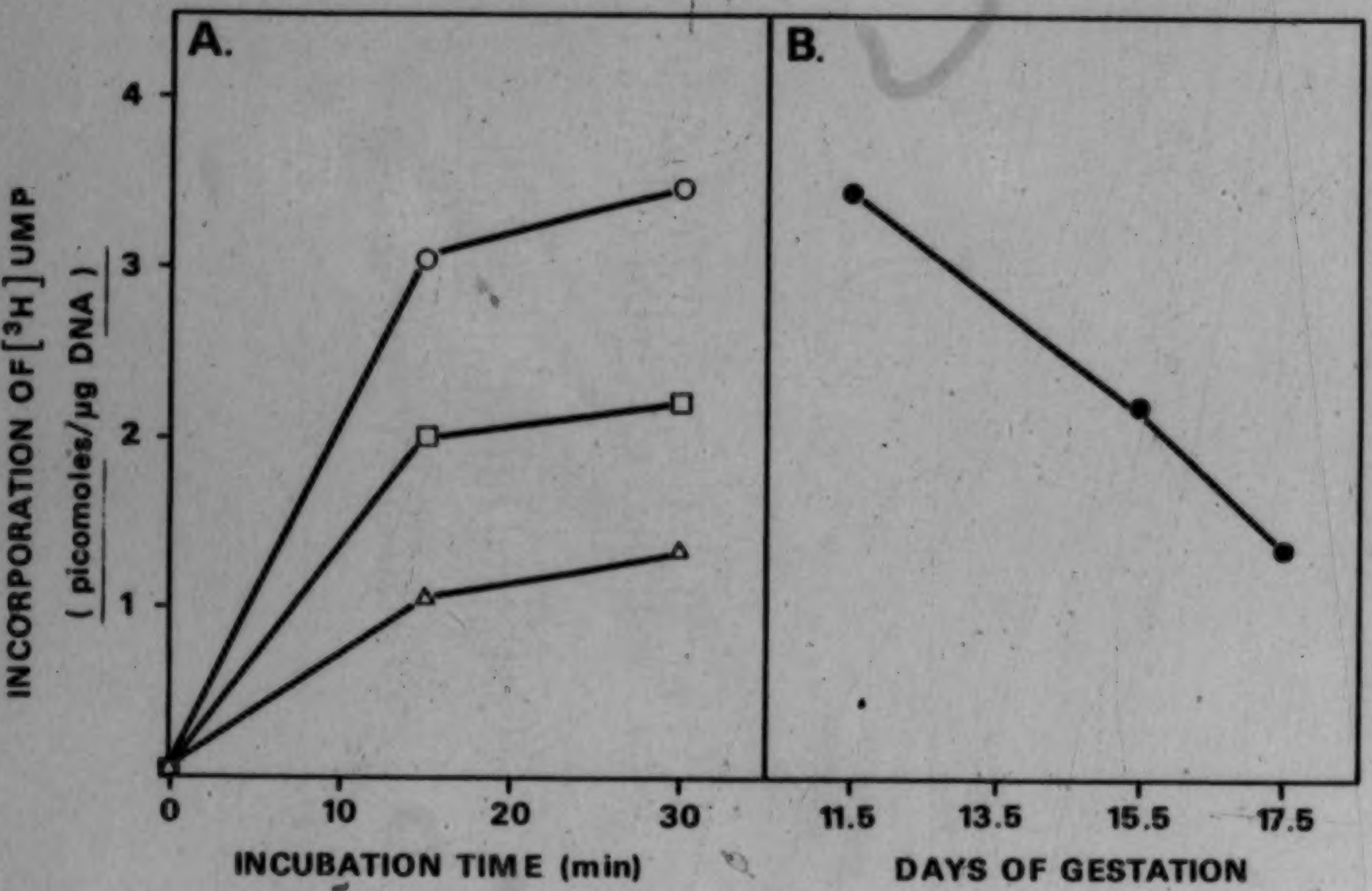
Table 1. Reduction in RNA synthesis in yolk sac explants between day 11.5 and 15.5

Experiment	RNA	$[^3H]$ UR INCORPORATED Day 11.5 (cpm/ug DNA)	$[^3H]$ UR INCORPORATED Day 15.5 (cpm/ug DNA)	$\frac{\text{Day 11.5}}{\text{Day 15.5}}$
I*	TOTAL	14,993	1,514	9.9
II	TOTAL	261,658	25,915	10.1
	POLY(A)$^+$	12,319	2,929	4.2
	POLY(A)$^-$	249,339	22,896	10.8

RNA was isolated from yolk sacs cultured with $[^3H]$ uridine for 4 hr as described in Materials and Methods. The RNA was fractionated by oligo d(T) cellulose chromatography into poly (A)$^+$ and poly (A)$^-$ RNA. The RNA samples were precipitated by the cold TCA method and radioactivity of each sample was determined.

* Results from Figure 6B.

Figure 7. RNA synthesis by nuclei in vitro. A. Nuclei were isolated from yolk sacs at different stages of gestation and incubated with [^{3}H] UTP under the conditions described in Materials and Methods. Incorporation of [^{3}H] UTP into TCA precipitable material was followed for 30 min using nuclei isolated at day 11.5 (○—○), day 15.5 (□—□), and day 17.5 (△—△). For each time point, the radioactivity was corrected for the zero time value (i.e. day 11.5, 1520 cpm, day 15.5, 1290 cpm, day 17.5, 1454 cpm). B. The 30 min time points of part A are re-plotted as a function of gestation.


A.
B.
INCORPORATION OF [³H] UMP
(picomoles/µg DNA)
4
3
2
1
0
10
20
30
INCUBATION TIME (min)
11.5
13.5
15.5
17.5
DAYS OF GESTATION

(Fig. 7A). In all cases, during the incorporation of UTP into RNA, there was an initial rapid increase to 15 min followed by a slow increase to 30 min. This behaviour is typical of [^{3}H] UTP incorporation by nuclei from various sources (26, 61). The secondary increase could be maintained for up to 50 min, after which there was no net isotope incorporation. Figure 7B shows the incorporation to 30 min as a function of gestation. There was a 1.6 fold decrease from day 11.5 to 15.5 and a 2.6 fold decrease from day 11.5 to day 17.5. These results indicate that nuclei at different days of gestation differ in their ability to synthesize RNA.

To further analyze the transcription by nuclei, the effects of α-amanitin and actinomycin D were studied (Table 2). α-Amanitin inhibits transcription of mRNA sequences, while actinomycin D inhibits all DNA dependent transcription. Under the incubation conditions used, more than 68% of the synthetic activity was sensitive to α-amanitin. The significance in the change in inhibition from day 15.5 to day 17.5 of 73% to 88% is not known. Studies by Ernest *et al.* in hen oviduct nuclei have shown that the remaining activity is due to rRNA synthesis (61). Actinomycin D decreased the incorporation of UTP by nearly 100%, indicating that the majority of the isotope incorporation was due to the transcription of DNA sequences.

(3) Protein Synthesis and Secretion During Gestation

(a) Total protein synthesis by yolk sac explants

The decrease in synthesis of mRNA during gestation (Table 1) may lead to a decrease in protein synthesis. The incorporation of [^{3}H] leucine and [^{35}S] methionine into protein was studied in yolk sac explants

Table 2. Effect of α-amanitin and actinomycin D on RNA synthesis by nuclei *in vitro*

INHIBITOR	DAYS OF GESTATION	[^{3}H] UMP INCORPORATION Control (cmp/ug DNA)	[^{3}H] UMP INCORPORATION Treated (cpm/ug DNA)	% INHIBITION
α-AMANITIN	11.5	1,627	518	68
	15.5	2,031	545	73
	17.5	1,200	147	88
ACTINOMYCIN D	11.5	1,627	97	94
	15.5	2,031	66	97
	17.5	1,200	126	90

Nuclei were isolated from yolk sacs at days 11.5, 15.5 and 17.5 of gestation and were incubated with [^{3}H] UTP as described in Materials and Methods. The inhibitors, α-amanitin and actinomycin D, were present at 2.5 μg/ml and 20 μg/ml, respectively, during the 30 min incubation. The counts were corrected for zero time value and percent inhibition was calculated as follows:

$$\% \text{ INHIBITION} = \frac{\text{CONTROL - TREATED}}{\text{CONTROL}} \times 100\%$$

collected at different days of gestation (Fig. 8). The kinetics of incorporation of the two isotopes by day 13.5 yolk sac explants were linear for at least 2 hr. Although similar results were obtained for other days of gestation, there were differences in absolute levels of incorporation. The incorporation of the two isotopes by yolk sacs at different stages of gestation showed similar patterns (Fig. 8C). Protein synthesis was relatively stable up to day 13.5 when there was a drastic decrease to day 15.5. At day 17.5, there was still 10 - 15% of the initial activity present, which was quite high when compared with the relative synthesis of DNA and RNA at this stage.

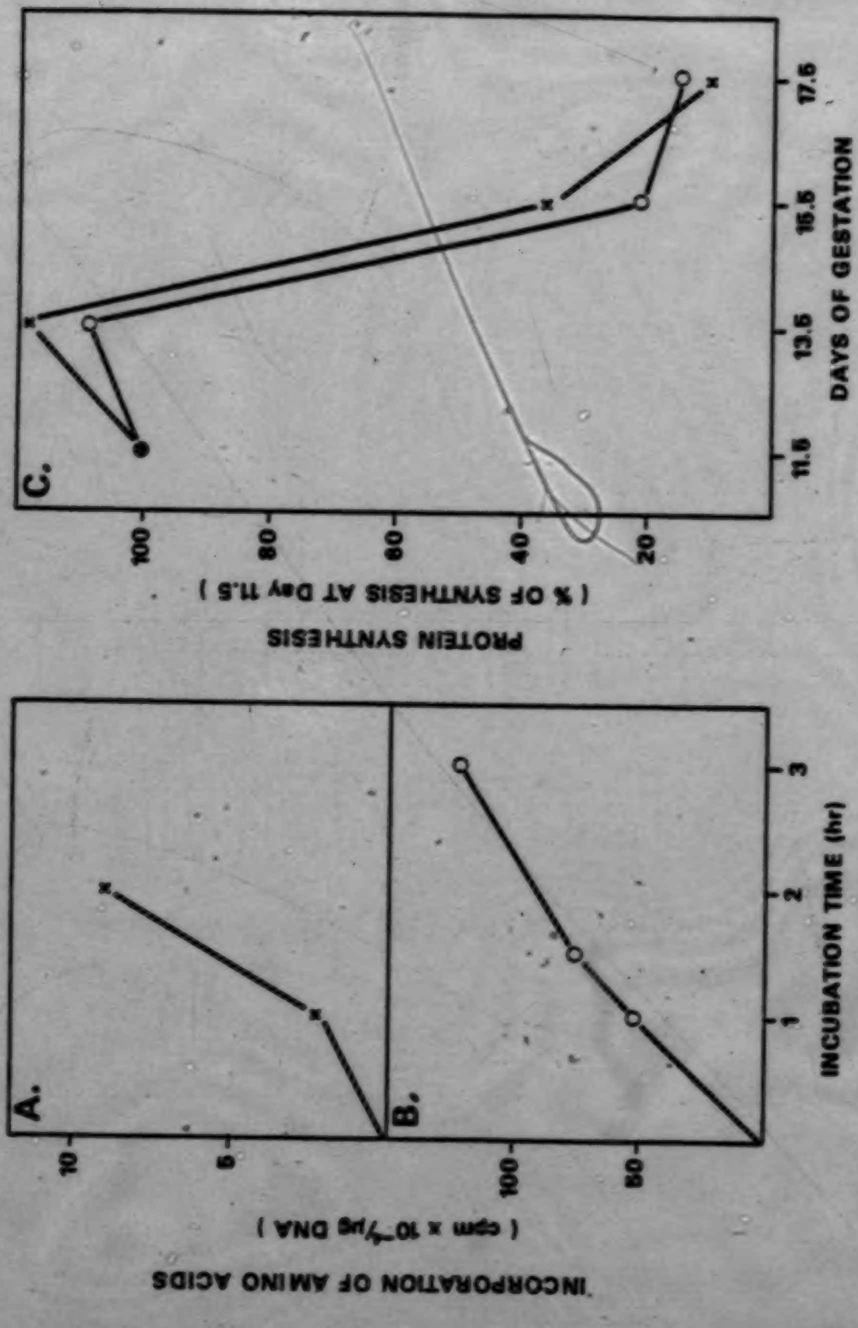
A comparison of protein synthesis (Fig. 8C) and RNA synthesis (Fig. 6B) showed that the decrease in RNA synthesis preceded the decline in protein synthesis by two days, suggesting that decreased RNA synthesis eventually leads to decreased protein synthesis. The decrease in poly $(A)^+$ RNA synthesis from day 11.5 to 15.5 was 4.5 fold (Table 1), which was similar to the decrease in ability to incorporate leucine (3.4 fold) and methionine (5.5 fold) during this same period. While there was low RNA synthesis at days 15.5 and 17.5, the protein synthesis observed may be accounted for in two possible ways. (1) Although less total RNA is being made, a greater proportion is mRNA. (2) As will be shown later, the types of mRNA's present at days 11.5 and 17.5 are very stable and are translated into the major proteins seen during this period.

(b) Synthesis of specific proteins

(i) SDS gel electrophoresis

Developmental changes in the synthesis of proteins by yolk sacs in short term organ culture were analyzed by SDS PAGE followed by

Figure 8. Protein synthesis in the yolk sac explants at different stages of gestation. Yolk sacs isolated from day 13.5 pregnant mice were cultured in the presence of (A) [^{3}H] leucine and (B) [^{35}S] methionine for increasing periods of time. Yolk sacs were lysed in the medium and a protein extract was prepared as described in Materials and Methods. Samples were assayed for incorporation of radioactive amino acid by hot TCA precipitation. (C) Yolk sacs were isolated from different stages of gestation and were incubated with [^{3}H] leucine for 2 hr, (X—X), or [^{35}S] methionine for 1 hr (O—O), and then assayed for hot TCA precipitable radioactivity. The 100% value was 1.29×10^6 cpm/μg DNA for [^{3}H] leucine incorporation and 5.3×10^5 cpm/μg DNA for [^{35}S] methionine incorporation.


A.
B.
C.
10
5
100
50
1
2
3
INCUBATION TIME (hr)
INCORPORATION OF AMINO ACIDS
(cpm x 10⁻⁴/µg DNA)
PROTEIN SYNTHESIS
(% OF SYNTHESIS AT Day 11.5)
100
80
60
40
20
11.5
13.5
15.5
17.5
DAYS OF GESTATION

fluorographic location of labelled proteins (Fig. 9). The pattern of protein synthesis at each stage of development was complex. A comparison of profiles from different developmental ages revealed that the majority of proteins were present in the same relative proportion throughout gestation, suggesting that their rates of syntheses were the same. However, most of these proteins represented a minor proportion of total protein synthesis. When comparing the more intense bands, significant developmental changes were observed. The most dramatic change occurred in AFP synthesis (for the identification of AFP see Fig. 14) which by day 15.5 accounted for a large proportion of the total protein synthesis. From day 9.5 to 13.5, the intensity of the AFP band increased greatly, then remained constant up to day 17.5, after which the band intensity decreased. There were several other proteins whose synthesis was greatly increased during this developmental period. An example is the protein with molecular weight of approximately 22,000. Before day 13.5, its relative proportion was very low but after day 13.5 it became a major protein. There were also several examples of proteins whose relative synthesis declined during development. The major example was a protein of approximate molecular weight 13,000 which was probably embryonic globin. The synthesis of this protein decreased drastically from day 11.5 to 13.5, which correlates exactly with the cessation of globin synthesis by primitive erythroid cells in the yolk sac (131).

(ii) Two dimensional gel electrophoresis

The proteins synthesized by yolk sac explants were analyzed by the two dimensional electrophoresis system of O'Farrell (150) (Fig. 10). This method separates the proteins according to two independent molecular

Figure 9. SDS gel electrophoresis of proteins synthesized in yolk sacs at different stages of gestation. Yolk sacs (5 or more) from different stages of gestation were cultured in methionine-free medium supplemented with 50 μCi/ml [^{35}S] methionine for 1 hr. Cultures were lysed and centrifuged at 100,000 xg for 60 min. Supernatants containing 38,000 cpm were analyzed on 8-16% linear gradient polyacrylamide SDS gels. The gels were fixed, prepared for fluorography and exposed to x-ray film for 4 days. Molecular weight markers were globin (15,000), chymotrypsinogen A (25,000), ovalbumin (45,000) and bovine serum albumin (70,000). The arrow indicates the band of marker AFP.

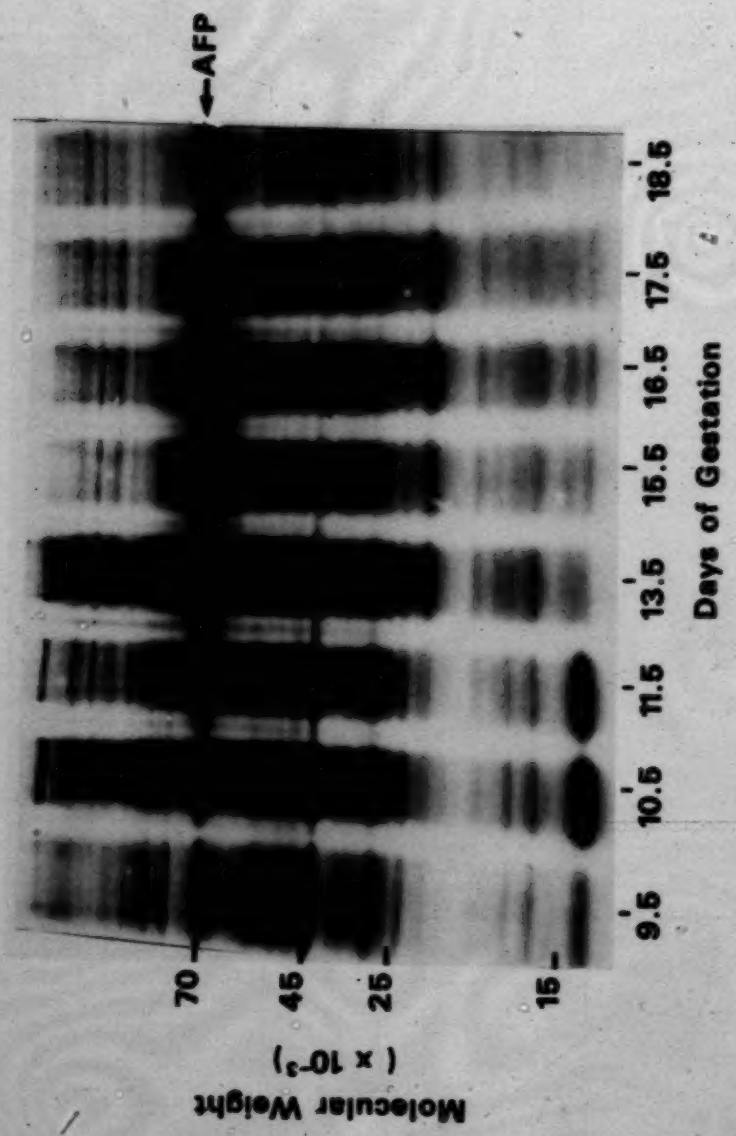
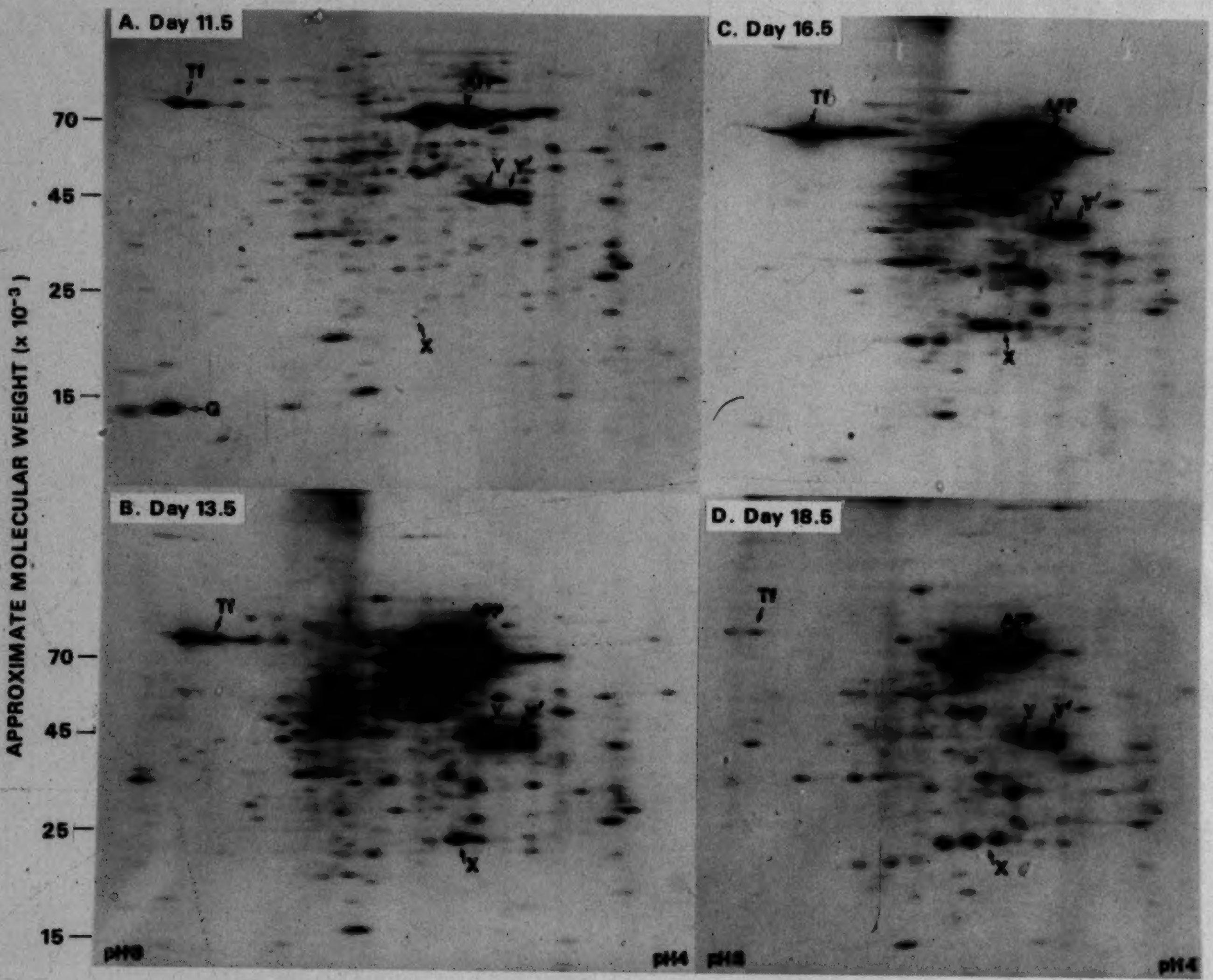

AFP
70
45
25
15
Molecular Weight
(x 10-3)
9.5
10.5
11.5
13.5
15.5
16.5
17.5
18.5
Days of Gestation

Figure 10. Two dimensional gel electrophoresis of proteins synthesized in the yolk sac at different days of gestation. Yolk sac proteins were labelled with [^{35}S] methionine as described in Materials and Methods. Samples containing 150,000 cpm were loaded onto isoelectric focussing gels and were focussed at 350 volts for 19 hr. The IEF gels were equilibrated in SDS sample buffer for 1 hr before they were applied to SDS polyacrylamide gels. After electrophoresis, the gels were fixed and prepared for fluorography. Radioactive protein spots were visualized by fluorography. The gels were exposed to the x-ray film for 6 days. AFP and Tf indicates the location of alphafetoprotein and transferrin. X, Y and Y' are unidentified proteins of interest, while the spot indicated by G is believed to be globin.



APPROXIMATE MOLECULAR WEIGHT (x 10⁻³)
70
45
25
15
A. Day 11.5
Tf
AFP
Y
Y'
X
G
B. Day 13.5
Tf
AFP
Y
Y'
X
pH8
pH4
C. Day 16.5
Tf
AFP
Y
Y'
X
D. Day 18.5
Tf
AFP
Y
Y'
X
pH8
pH4

parameters, isoelectric point and molecular weight. In each case, the fluorogram shows a large number of unique proteins. AFP was the most intense spot on each gel (for identification of AFP and Tf see Fig. 15) and appeared to be more of a streak than an individual spot. AFP consists of a number of different isoelectric forms which under the long exposure times blurred together, forming a streak. Transferrin (Tf) was also a major spot, especially at day 16.5. It also seemed to have a number of isoelectric forms, and these seemed to change during gestation. Tf (80,000 M.W.) could not be resolved from AFP on one dimensional SDS gels but because the two proteins had different pI's, they could be easily separated on O'Farrell gels. Both AFP and Tf seemed to change in quantity during gestation with maximum intensity of the spots being at day 16.5. Protein X, found at molecular weight of approximately 25,000, was not detected at day 11.5 but increased in amount and also in heterogeneity to day 17.5. Its identity was unknown. The pattern of change in this protein was very similar to AFP and Tf, and these three proteins may be an example of coordinate gene expression. The doublet, Y and Y', at 45,000 molecular weight, might at first be considered to be one spot, but at day 16.5, the Y' spot appeared to shift to a higher molecular weight. This was even more pronounced at day 17.5 where spot Y was reduced in intensity. These spots had the same molecular parameters as actin. Spot G was believed to be identical with the intense band at molecular weight 13,000 on one-dimensional SDS gels (Fig. 9) and was tentatively identified as embryonic globin.

The conditions of fluorography used here allowed the major proteins synthesized by the yolk sac to be visualized. Using a longer exposure the number of minor spots increased, suggesting that there were many more

6

proteins being made but at relatively low abundancies.

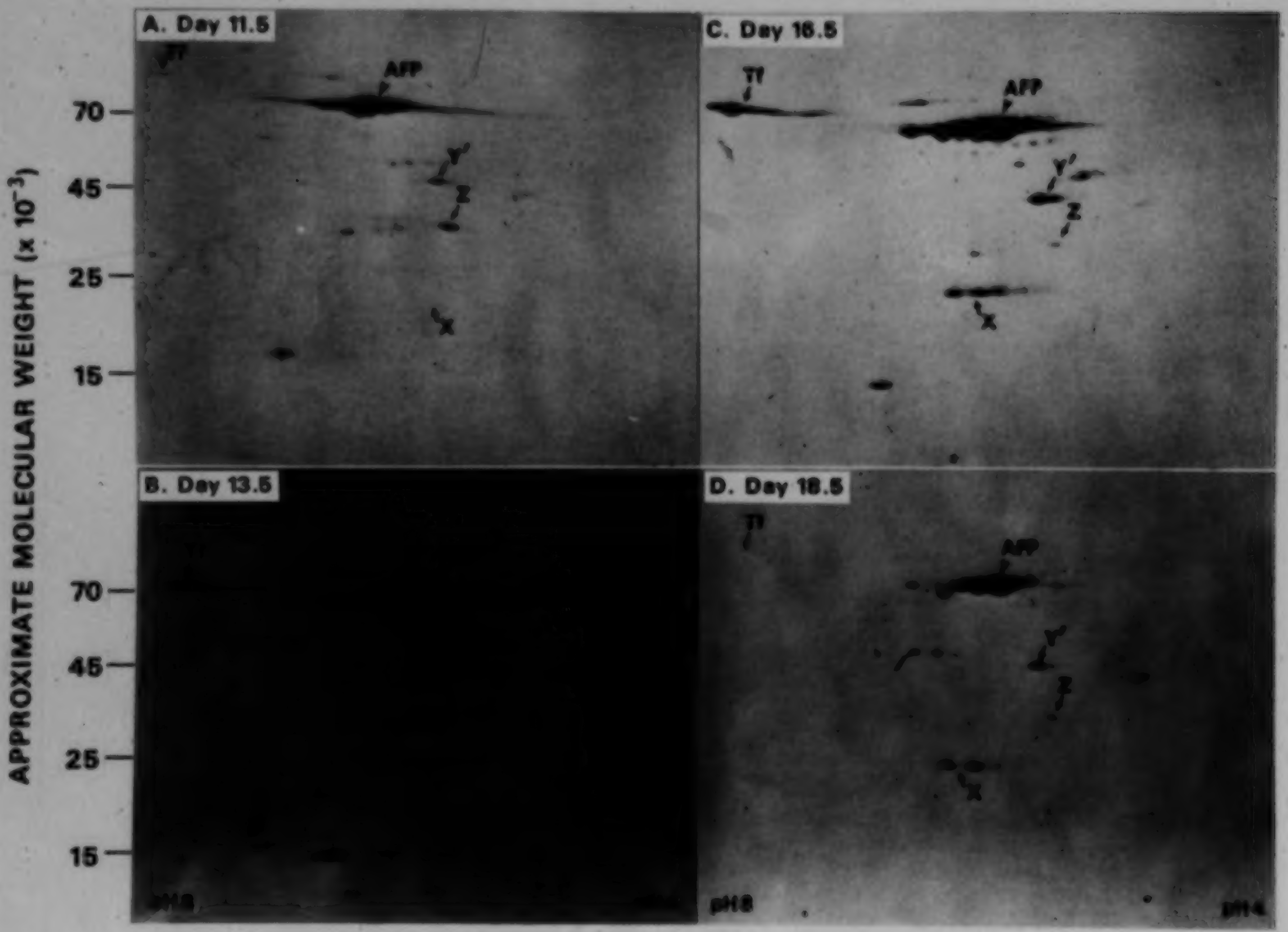
These results indicated that the yolk sac makes a large number of proteins, including a few major ones whose relative rates of synthesis change during gestation.

(c) Protein secretion by yolk sac explants

(i) Two dimensional gel analysis

AFP is found in the fetal and maternal serum and in the amniotic fluid. This suggests that the yolk sac and/or the liver secrete AFP. To see if the yolk sac secretes AFP and other proteins, yolk sacs were cultured in the presence of [^{35}S] methionine and the radioactive proteins found in the medium were analyzed by two dimensional gel electrophoresis (Fig. 11). At each gestational day, only a small number of proteins were found in the secreted fraction, suggesting a selective release of certain proteins from the yolk sacs. AFP was the major secreted protein at each day of gestation. Although the different isoelectric forms of AFP were still poorly separated, there was an increase in the intensity of the acidic forms of AFP with gestation, especially when AFP secreted at day 11.5 is compared to that secreted at day 18.5. By superimposing the fluorograms of the cellular (Fig. 10) and secreted proteins (Fig. 11), one can identify which proteins are secreted. Tf and protein X are both secreted and their pattern of spots is similar to that found synthesized in the yolk sac. Protein Y' was the only one of the doublet Y-Y' that was secreted. Protein Z was a secreted protein which was decreasing in relative abundance during gestation. The synthesis of this protein decreased during gestation (Fig. 10) but was not originally noticed as it was not a major protein. Several other

Figure 11. Protein secretion by yolk sac explants at different stages of gestation. Yolk sacs from different gestational days were cultured in [^{35}S] methionine containing medium for 1 hr at 37°C. The yolk sacs were removed and radioactive protein in the medium was analyzed by two dimensional gel electrophoresis. Samples contained 50,000 cpm hot TCA precipitable radioactivity. The radioactive spots were visualized by fluorography with an exposure time of 18 days. AFP indicates the position of alphafetoprotein and Tf, the position of transferrin. X, Y, Y' and Z are unidentified proteins of interest.


APPROXIMATE MOLECULAR WEIGHT (x 10^{-3})
70
45
25
15
A. Day 11.5
Tf
AFP
Y'
Z
X
C. Day 16.5
Tf
AFP
Y'
Z
X
B. Day 13.5
pH8
D. Day 18.5
Tf
AFP
Y'
Z
X
pH8
pH4

minor proteins were secreted. The yolk sac secretes several proteins of which the major ones go through gestational changes, and these changes are directly related to their synthesis in the yolk sac.

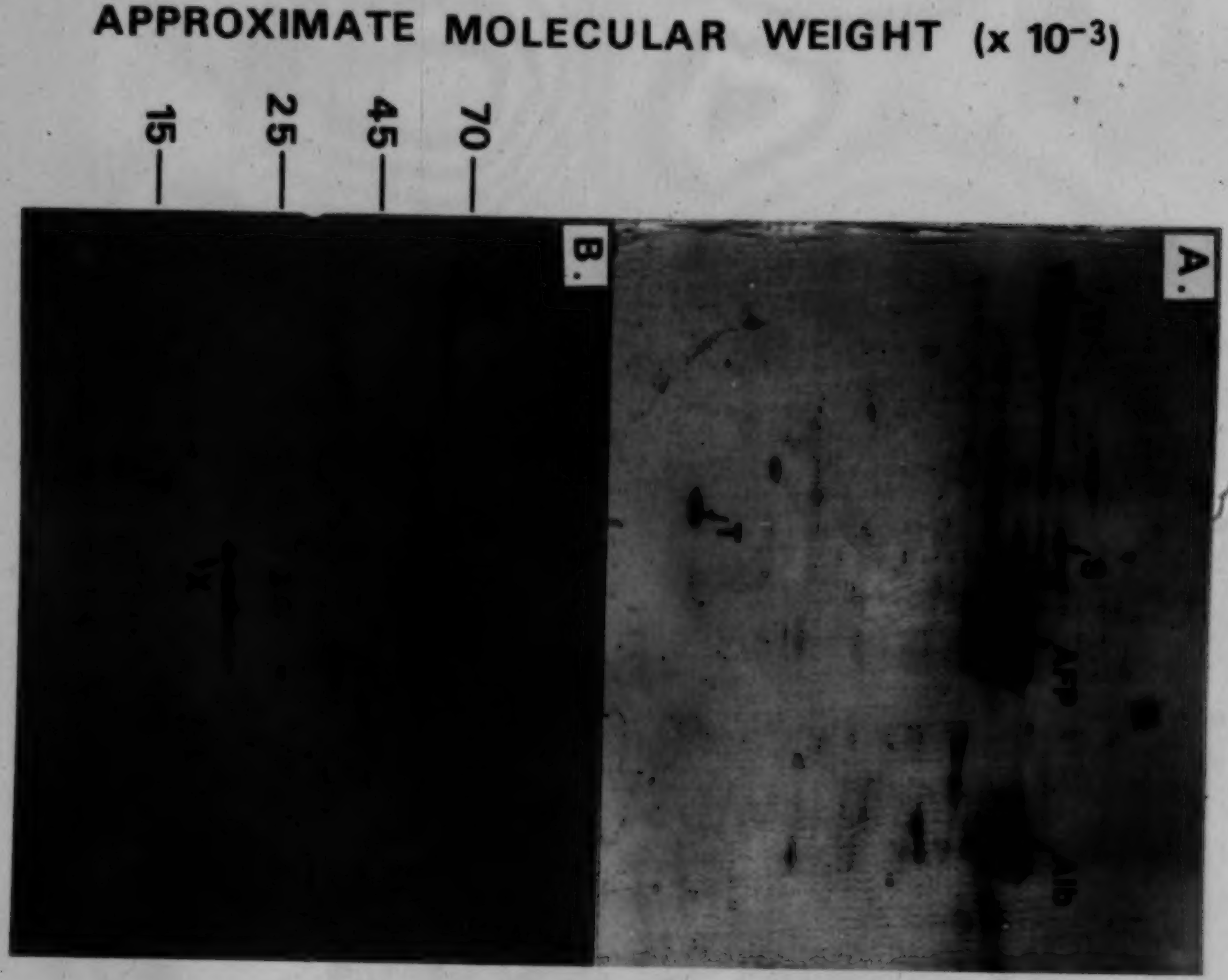
(ii) A comparison of total amniotic fluid protein to proteins secreted by the yolk sac explants

The major protein components of mouse amniotic fluid are AFP (50%), albumin (36%), and transferrin (14%) (78). Amniotic fluid is contained within the yolk sac during the last half of gestation. The proteins synthesized and secreted by the yolk sac could contribute to the proteins found in the amniotic fluid. A comparison of 16 day amniotic fluid protein and proteins secreted by day 16.5 yolk sacs *in vitro* was made (Fig. 12). The amniotic fluid showed three major spots which are Tf, AFP and albumin. These were present in the approximate ratio stated above and constituted more than 95% of the protein present. Several other proteins were present but in minor quantities relative to Tf, AFP and albumin. The proteins secreted by the yolk sac included large quantities of AFP and Tf, but little, if any, albumin. Amongst the minor protein spots, only three proteins appeared to be common to both amniotic fluid and yolk sac (R, S and T). The proteins X and Y which are secreted by yolk sacs *in vitro* were not present in equivalent amounts in the amniotic fluid.

C. Discussion

The results shown above indicate that macromolecular synthesis changes drastically in the yolk sac during the last half of gestation. Both DNA and RNA synthesis decrease in a similar fashion and at day 17.5 are essentially zero. The decrease in protein synthesis occurs two

Figure 12. Comparison of total amniotic fluid proteins to proteins secreted by the yolk sac in vitro. A. Amniotic fluid was isolated from several 16.0 day conceptuses by puncturing each intact yolk sac with a syringe needle and removing only the clear fluid surrounding the fetus. The fluid was centrifuged in an Eppendorf microfuge and frozen. Protein content was determined by the BioRad Protein Assay Kit. Approximately 200 µg of amniotic fluid protein were analyzed by two dimensional gel electrophoresis. The gel was stained with Coomassie blue, destained and photographed. B. Proteins synthesized and secreted by the 16.5 day yolk sac explants were analyzed by two dimensional electrophoresis and radioactive spots were visualized by fluorography.


APPROXIMATE MOLECULAR WEIGHT (x 10⁻³)
70
45
25
15
A.
B.
S
AFP
Alb
T
X

days after the decline in RNA and DNA synthesis but even at late stages was maintained at approximately 10% of the activity at day 11.5.

UTP incorporation into RNA by yolk sac nuclei decreased 1.6 fold from day 11.5 to 15.5 and 2.6 fold from day 11.5 to 17.5. RNA polymerase II activity predominated (Table 2) indicating that incorporation was mostly into mRNA-like molecules. The decrease in poly $(A)^+$ RNA synthesis by yolk sac explants from day 11.5 to 15.5 was 4 fold (Table 1) which is similar to the decrease in RNA synthesis by nuclei during this period (Fig. 7). Therefore the contribution by changes in UTP pool size to the decrease in RNA synthesis appears to be small, and the decrease in RNA synthesis during gestation seems to be due ,at least in part, to differences in the transcriptional ability of the yolk sac.

The electrophoretic analysis of the total protein synthesis (Figs. 9 and 10) showed that there are only a few changes in the species of proteins synthesized during gestation with a large proportion of the protein synthesized remaining constant. This indicates that the same genes are being transcribed at a reduced rate late in gestation and no major changes have occurred in the types of genes that are being transcribed.

The changes in protein synthesis resulted in a group of four proteins: AFP, Tf, X and Y' becoming the major portion of the protein synthesis by day 16.5. This group of proteins is secreted by the yolk sac. The preferential synthesis and secretion of these proteins suggests they may have an important function in fetal development.

AFP, Tf and a few of the minor proteins secreted from the yolk sac were present in the amniotic fluid. Since albumin is present in the amniotic fluid, AFP and Tf could arise from both the yolk sac and liver.

Ruoslahti suggests that up to 65% of the amniotic fluid AFP may be due to yolk sac synthesis, indicating that the yolk sac is a major source of AFP, Tf and possibly the other minor components found in the amniotic fluid (174). The reason for the absence of X and Y' proteins is not clear at present, but they may be released either into the uterine cavity or into the intercellular space of the yolk sac where they remain.

In summary, the results show that DNA, RNA and protein synthesis decrease near the end of gestation. During the fall in protein synthesis, a small group of proteins, of which AFP is the predominant member, constitutes a major fraction of the total protein synthesis. These proteins are secreted by the yolk sac and several of these proteins appear in the amniotic fluid.

The biochemical changes that take place in the yolk sac during gestation are remarkably similar to events that occur during embryonic erythropoesis (131). In embryonic erythropoetic cells, the cessation of cell division occurs concurrently with a decrease in RNA and protein synthesis. During the period of decreased protein synthesis, globin becomes the predominant protein synthesized. Both the fetal erythrocyte and the yolk sac are present only during fetal development and appear to go through a process of terminal differentiation.

CHAPTER IV

AFP: SYNTHESIS, SECRETION AND GLYCOSYLATION BY YOLK SAC DURING GESTATION

A. Introduction

In this chapter, the identification of AFP and Tf by immunoprecipitation followed by analysis on two dimensional gels is described. AFP synthesis was quantified as a function of gestation using immunoprecipitation. This type of analysis was also used to study the secretion of AFP and modifications that occur during gestation.

Glycosylation of AFP and its possible role in the secretion of AFP were studied using tunicamycin. Tunicamycin inhibits glycosylation by interfering with the formation of the lipid-oligosaccharide intermediate formed during N-linked glycosylation (91). The sialylation of AFP is believed to increase during gestation, giving rise to the changes in electrophoretic microheterogeneity (128). The changes in the microheterogeneity of the cellular and secreted forms of AFP are shown.

B. Results

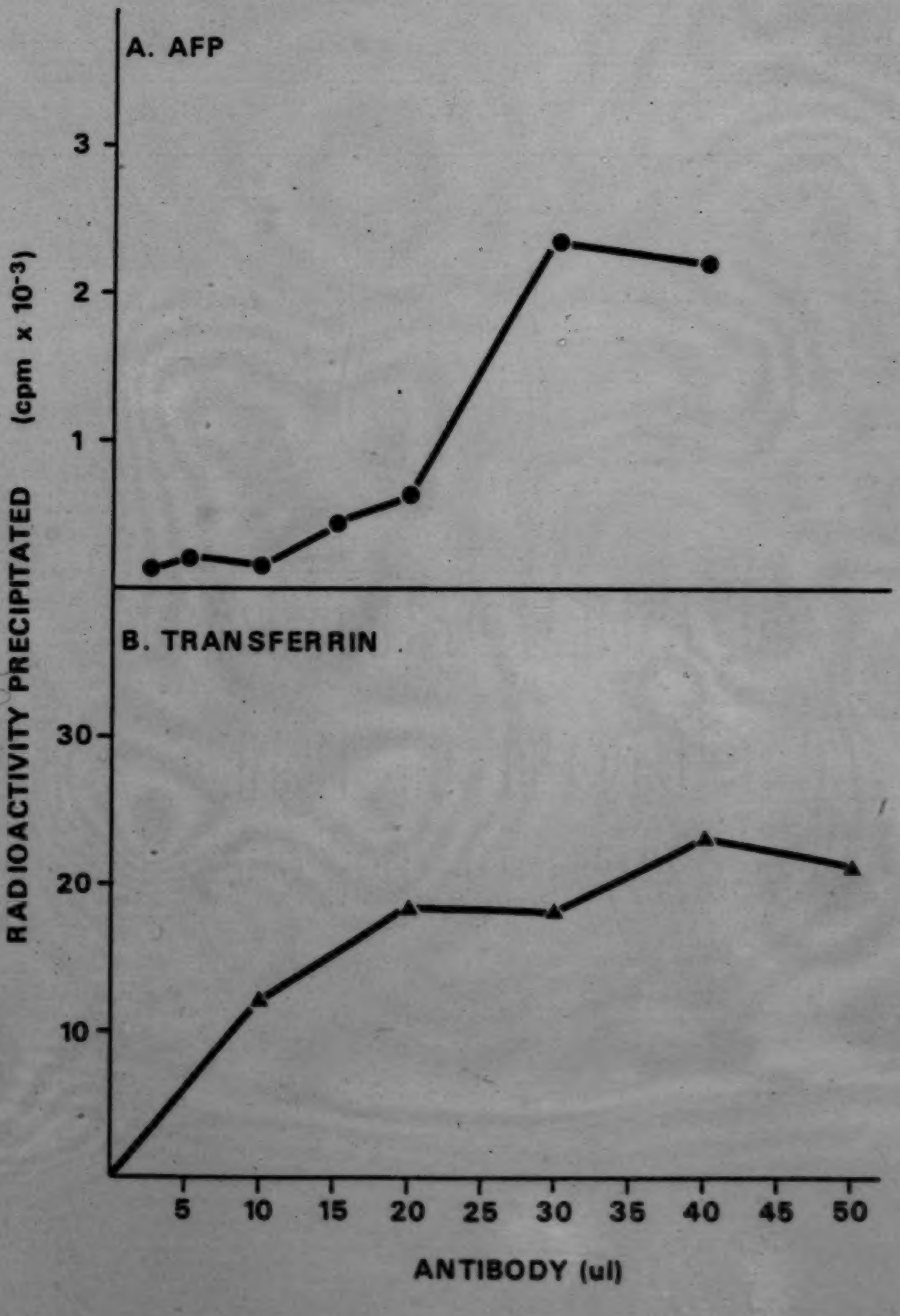
(1) Identification of AFP and Transferrin

(a) Immunoprecipitation

Antibodies specific for AFP and Tf were obtained as described in Materials and Methods. AFP and Tf in yolk sac lysates were titrated using increasing amounts of antibodies and constant amount of carrier antigens (Fig. 13). The conditions for the quantitative precipitation

Figure 13. Titration of AFP and Tf with specific antibody.

A. AFP: Yolk sacs from a 13.5 day pregnant mouse were cultured in medium containing [^{3}H] leucine and a lysate was prepared as described in Materials and Methods. Increasing amounts of rabbit anti-AFP (90 mg/ml) were added to tubes containing 200 µl of lysate and 8500 cpm of hot TCA precipitable material. Carrier AFP (3 µg) was added to each tube and the tubes were incubated at 28°C for 1 hr and at 4°C overnight. The precipitates were washed, dissolved in NCS (Amersham) and the radioactivity was determined. B. Tf: Yolk sacs from a 15.5 day pregnant mouse were cultured in medium containing [^{35}S] methionine and a lysate was prepared. Fifty µl of lysate, which contained 1.4 x 10^6 cpm were added to each tube with 10 µl of carrier Tf solution. Anti-Tf was added and the mixture was incubated at 28°C for 1 hr and then at 4°C overnight. The precipitates were washed and dissolved in SDS buffer and the radioactivity in 5 µl was determined.


A. AFP
B. TRANSFERRIN
RADIOACTIVITY PRECIPITATED (cpm x 10-3)
3
2
1
30
20
10
5
10
15
20
25
30
35
40
45
50
ANTIBODY (ul)

of AFP were shown to be 30 µl of this antibody preparation in the presence of 3 µg of AFP (Fig. 13A). In all of the following studies, 40 µl of antibody was used to precipitate 200 µl of yolk sac lysate. For Tf (Fig. 13B), 40 µl of antibody were required to precipitate all the Tf from 50 µl of day 15.5 yolk sac lysate plus carrier Tf.

(b) Electrophoretic analysis

The immunoprecipitates were dissolved in SDS sample buffer and analyzed along with the original sample on SDS polyacrylamide gels (Fig. 14). There were a number of radioactive bands present in the total secreted fraction (slot 1) which corresponded in molecular weight to the major spots seen in the two dimensional gels (Fig. 11, Chapter III). The immunoprecipitate of AFP (slot 2) showed only one band which corresponds to the major band in the secreted fraction. The same major band could also be immunoprecipitated from the cellular fraction using this antibody (results not shown). The total cellular fraction (slot 3) had a complex pattern of protein bands but only one band could be precipitated from this fraction using Tf antibody. On this gel, AFP and Tf were distinct from each other and a comparison of the cellular and secreted fractions revealed the presence of both proteins. This indicates that Tf is secreted. These proteins constitute a major portion of the protein that is synthesized and secreted by the yolk sac.

The immunoprecipitates were analyzed by two dimensional electrophoresis (Fig. 15). There were two major spots in each photograph of the stained gels; one is the heavy chain of the IgG molecule at 50,000

Figure 14. SDS gel analysis of AFP and Tf immunoprecipitates.

Yolk sacs at day 15.5 were cultured in the presence of [^{35}S] methionine, and cellular and secreted fractions were prepared as described in Materials and Methods. The secreted (23,000 cpm, slot 1) and the cellular fractions (72,000 cpm, slot 3) were analyzed on a 10% polyacrylamide gel containing 0.1% SDS (see Materials and Methods). Tf and AFP were precipitated from the cellular and secreted fractions respectively. The immunoprecipitates (10,000 cpm AFP and 9,000 cpm transferrin) were analyzed on the same SDS slab gel (slots 2 and 4 respectively). The gel was subjected to fluorography for 30 hr to visualize the radioactive bands.

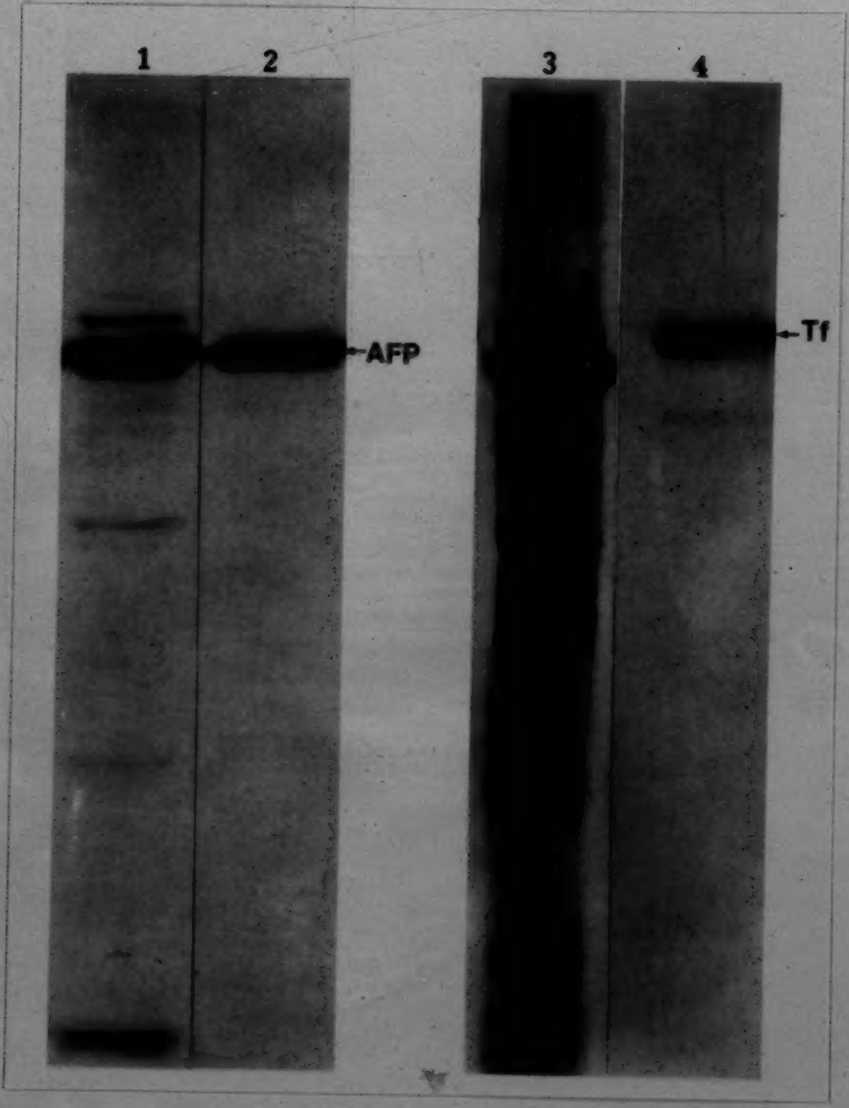
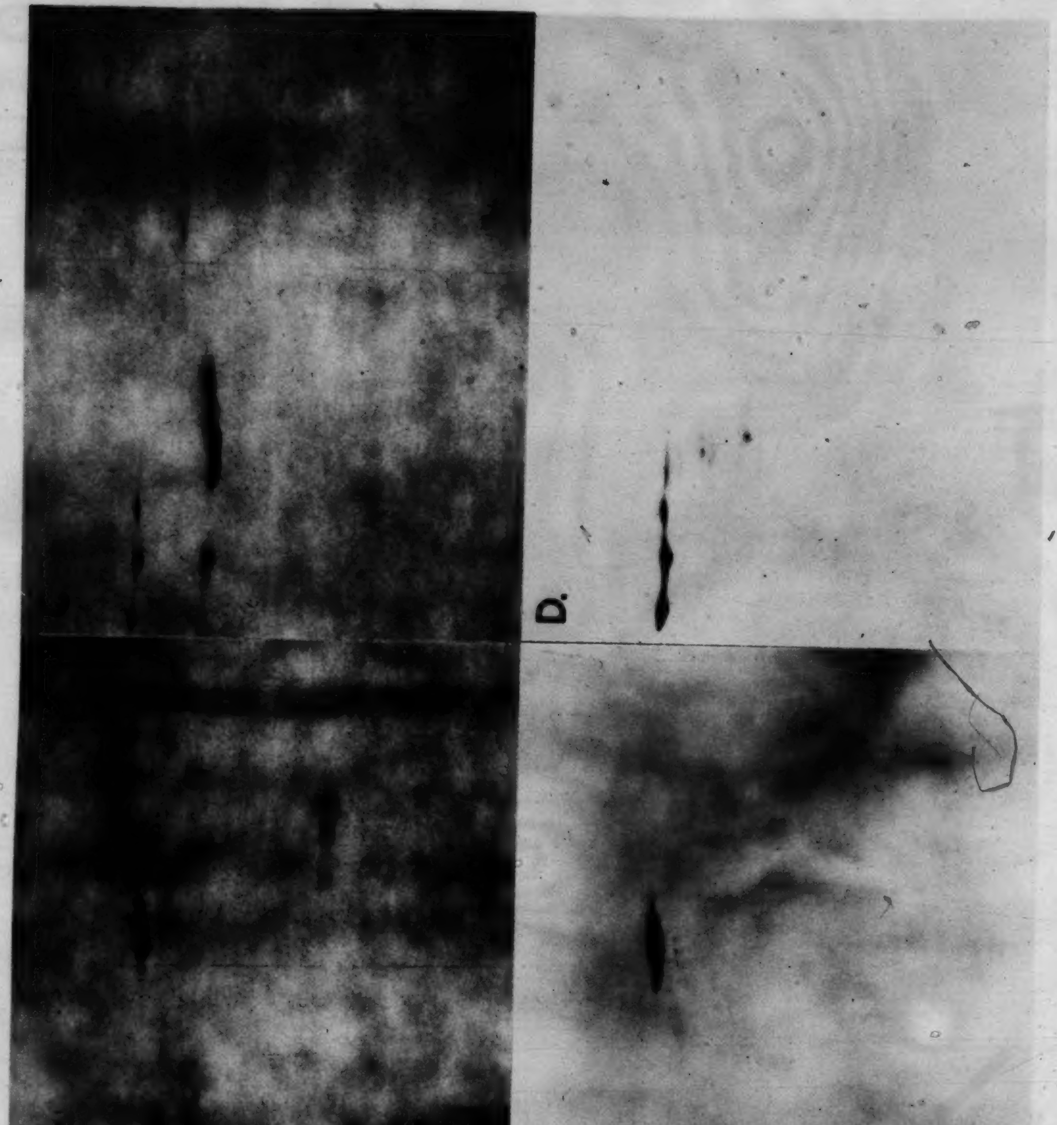

1
2
3
4
AFP
Tf

Figure 15. Two dimensional electrophoresis of AFP and Tf immunoprecipitates

The secreted fraction of yolk sac cultures was precipitated with anti-AFP while the cellular fraction was precipitated with anti-Tf as described in Materials and Methods. The immunoprecipitates were analyzed by two dimensional electrophoresis as described in Materials and Methods except that the samples were made up directly in urea, Np-40 2-mercaptoethanol and ampholines. The gels were then stained with Coomassie blue, destained and photographed. They were then prepared for fluorography and exposed to x-ray film for 18 hr in the case of AFP and 15 hr for Tf. (A) Photograph of the stained immunoprecipitate of AFP. (B) Fluorogram of the immunoprecipitated AFP. (C) Photograph of the stained Tf immunoprecipitate. (D) Fluorogram of the immunoprecipitated Tf.


D.
B.

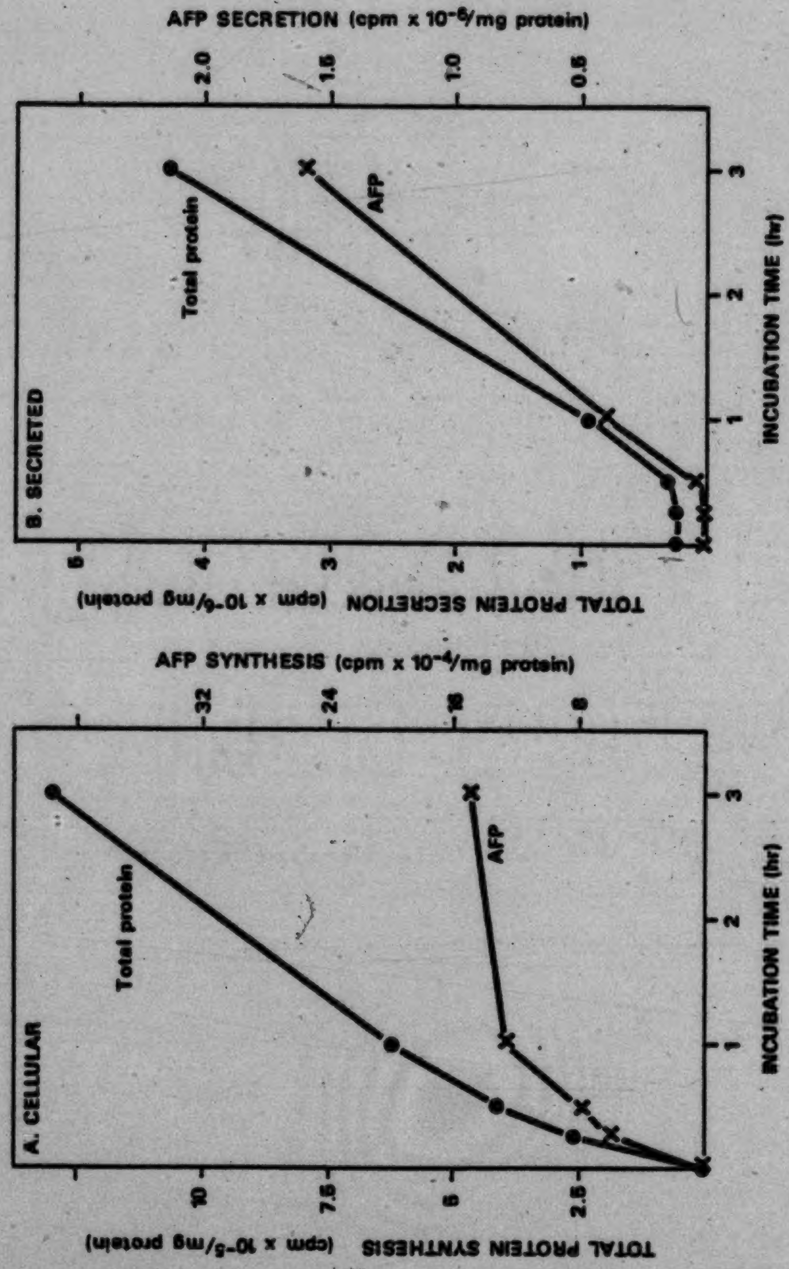
molecular weight, and the other, AFP (Fig. 15A) and Tf (Fig. 15C). The light chain of the IgG molecule was not visible in these photographs. In the fluorograms of these gels, only the AFP (Fig. 15B) and Tf (Fig. 15D) spots were radioactive, indicating that the yolk sac synthesizes AFP and Tf. The patterns of both proteins were heterogeneous, showing that the antibody could recognize and precipitate the different forms. The specificity of both the antibody and the immunoprecipitation conditions was shown on these gels by the lack of other radioactive spots. The location and intensities of the AFP and Tf spots were then used to identify these proteins in the total cellular and secreted protein fraction which were analyzed by two dimensional gel electrophoresis (Figs. 10 and 11).

(2) AFP Synthesis and Secretion

(a) Kinetics of synthesis and secretion

The appearance of radioactive protein in the cellular fraction after the addition of isotope, and the subsequent secretion of labelled protein into the medium, are shown (Fig. 16). By 15 min after the addition of [^{35}S] methionine to the medium, radioactive protein and AFP were both found in the yolk sac cells (Fig. 16A). Isotope incorporation into total protein was essentially linear up to 3 hr, while AFP synthesis increased for about 1 hr and then seemed to level off. Analysis of the appearance of radioactive total protein and AFP in the medium showed that until 30 min there was no significant secretion of protein, after which both total protein and AFP were secreted in a linear fashion for 3 hr (Fig. 16B).

Figure 16. Kinetics of AFP synthesis and secretion. Yolk sacs from day 15.5 were cultured in medium containing 25 μCi/ml[^{35}S] methionine for increasing periods of time. (A) Lysates were prepared from the isolated yolk sacs. Total protein, hot TCA precipitable radioactivity, and the anti-AFP precipitable radioactivity were determined for each time point. Each sample had the zero time value subtracted. (0—0) specific activity of total protein, (x—x) specific activity of AFP. (B) The medium containing the secreted proteins was clarified by centrifugation and analyzed for total protein content, labelled protein, and radioactive AFP. (0—0) total secreted protein, (x—x) secreted AFP.


A. CELLULAR
Total protein
AFP
TOTAL PROTEIN SYNTHESIS (cpm x 10⁻⁵/mg protein)
2.5
5
7.5
10
AFP SYNTHESIS (cpm x 10⁻⁴/mg protein)
8
16
24
32
INCUBATION TIME (hr)
1
2
3
B. SECRETED
Total protein
AFP
TOTAL PROTEIN SECRETION (cpm x 10⁻⁶/mg protein)
1
2
3
4
5
AFP SECRETION (cpm x 10⁻⁶/mg protein)
0.5
1.0
1.5
2.0
INCUBATION TIME (hr)
1
2
3

(b) Changes in synthesis and secretion with gestation

Gestational changes in AFP synthesis relative to total protein synthesis were determined by quantitative immunoprecipitation of labelled yolk sac cellular proteins (Fig. 17). AFP constituted a large proportion of the total protein synthesized during the last half of gestation. Initially, AFP synthesis represents only about 1.5% of total labelled cellular protein, but increases dramatically to day 15.5 when AFP represents 22% of the total protein synthesized The relative rate of AFP synthesis then declined to 8.5% by day 18.5. This result is similar to the electrophoretic results (Figs. 9 and 10), which showed qualitatively that AFP was a major protein from days 11.5 to 18.5.

To determine the relative proportions of AFP secretion at different stages of gestation, the area of each of the radioactive spots in Fig. 11 was measured and the percent AFP of the total area was calculated (Fig. 18). AFP constitutes approximately half of the total secreted protein and this does not change significantly during gestation.

(3) Glycosylation of AFP

(a) Glycosylated proteins in the yolk sac

AFP found in serum and amniotic fluid is glycosylated. To see if the yolk sac synthesized glycosylated proteins including AFP, yolk sacs were cultured in the presence of [^{3}H] glucosamine. The labelled protein found in the cellular fraction was analyzed by SDS gel electrophoresis (Fig. 19A). After an exposure of 30 hr (slot 1), only one band was present and it was in the AFP region. If the gel was exposed for 28 days (slot 2), many more bands were revealed. However AFP was still the most intensely labelled protein. The glycosylated proteins were

Figure 17. Developmental changes in the relative synthesis of AFP. Yolk sacs from different stages of gestation were cultured in medium containing [^{35}S] methionine for 1 hr at 37°C. The yolk sacs were isolated and lysed. The lysates were analyzed for total protein synthesis and AFP synthesis. AFP synthesis relative to total protein synthesis was calculated directly.

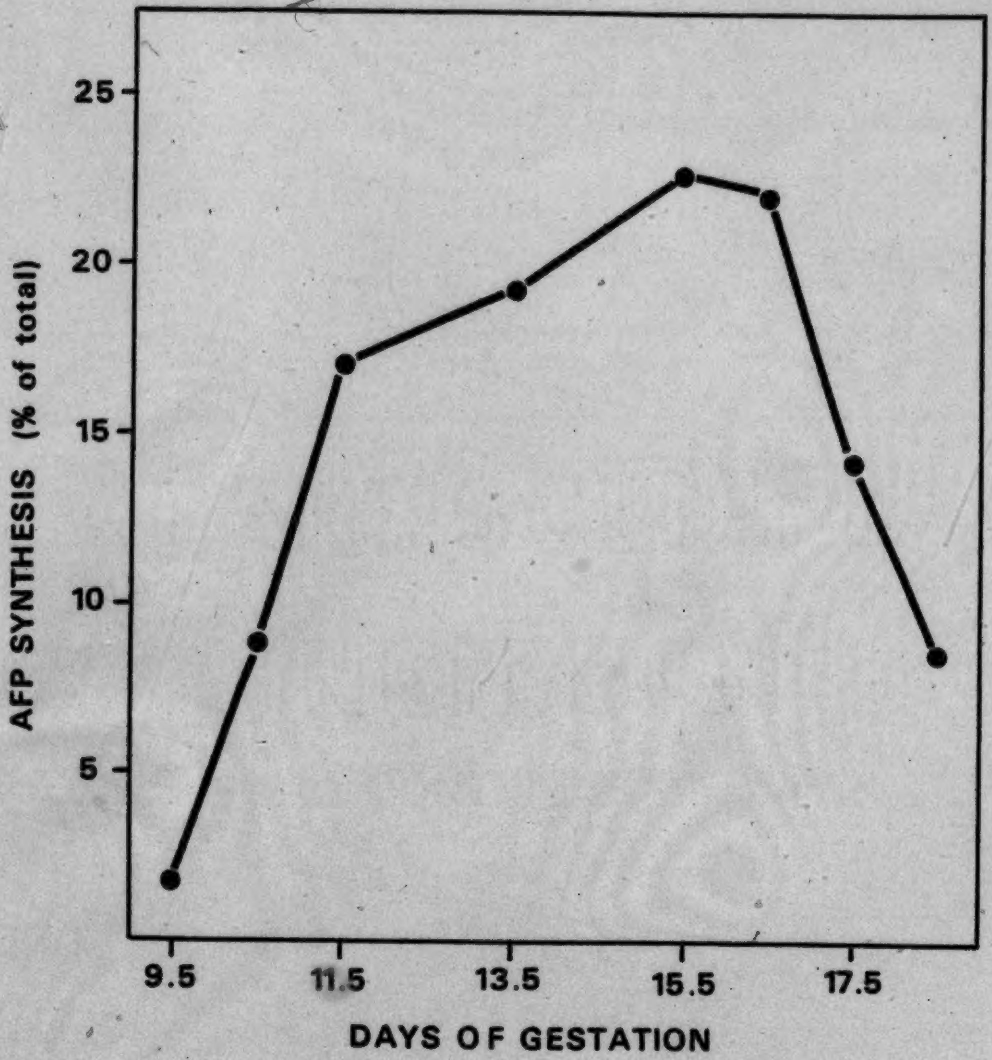

AFP SYNTHESIS (% of total)
25
20
15
10
5
9.5
11.5
13.5
15.5
17.5
DAYS OF GESTATION

Figure 18. AFP secretion during development. The medium in which the yolk sacs were cultured for 1 hr (Fig. 17) was clarified by centrifugation and analyzed by two dimensional gel electrophoresis (Fig. 11). The relative proportion of AFP at each day of gestation was determined by measuring the area of the radioactive spots using a planimeter and calculating the area of the AFP spot as a percent of the total area.

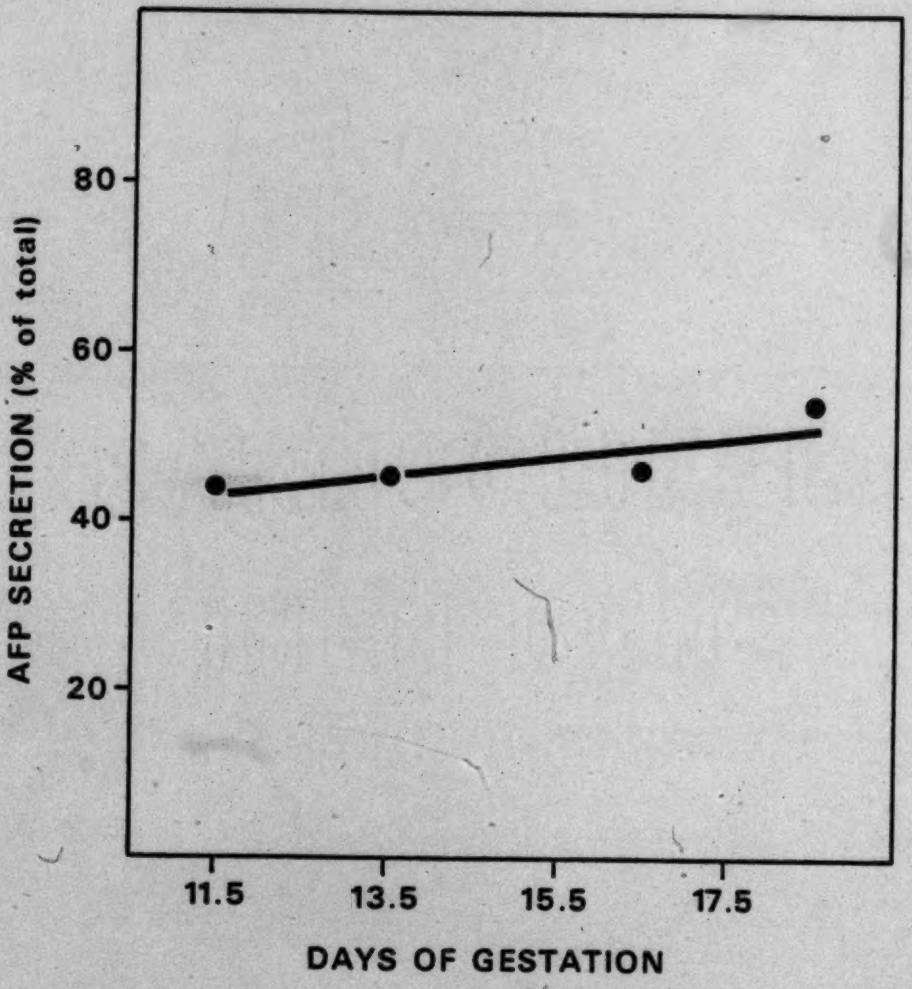
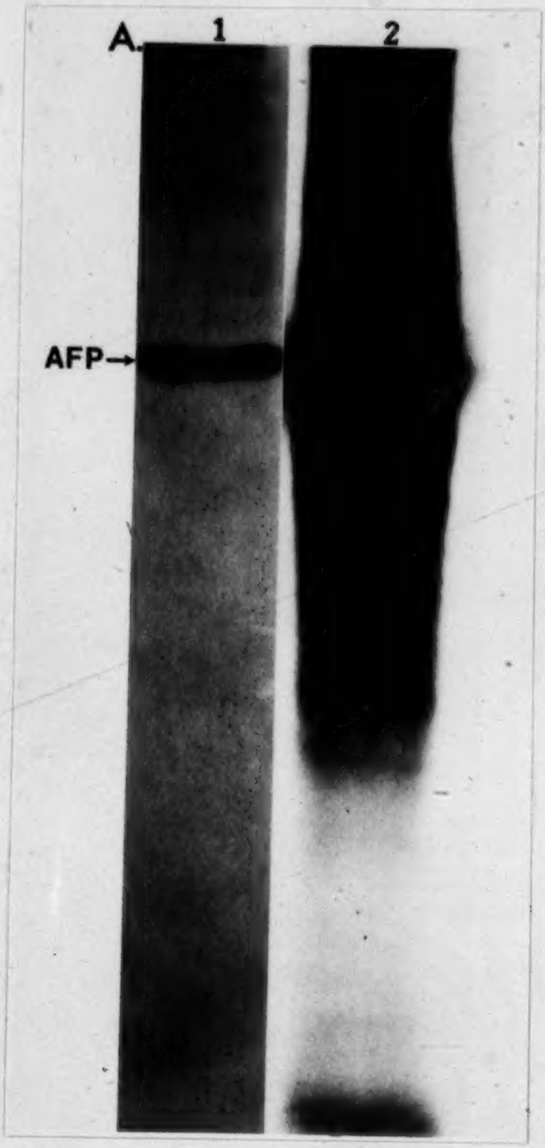

AFP SECRETION (% of total)
80
60
40
20
11.5
13.5
15.5
17.5
DAYS OF GESTATION

Figure 19. Incorporation of [^{3}H] glucosamine into glycoproteins in yolk sac. Yolk sacs at day 15.5 were isolated and cultured in the presence of [^{3}H] glucosamine. The yolk sacs were removed and lysed. The lysate was analyzed for incorporation of [^{3}H] glucosamine into total protein. (A) A sample containing 42,000 cpm was analyzed on an SDS slab gel. The gel was prepared for fluorography and exposed to x-ray film for (1) 30 hr and (2) 28 days. The arrow indicates the position of purified AFP. (B) A sample containing 83,000 cpm was analyzed by two dimensional electrophoresis and the glycoproteins detected by fluorography for 28 days.


A.
1
2
AFP→

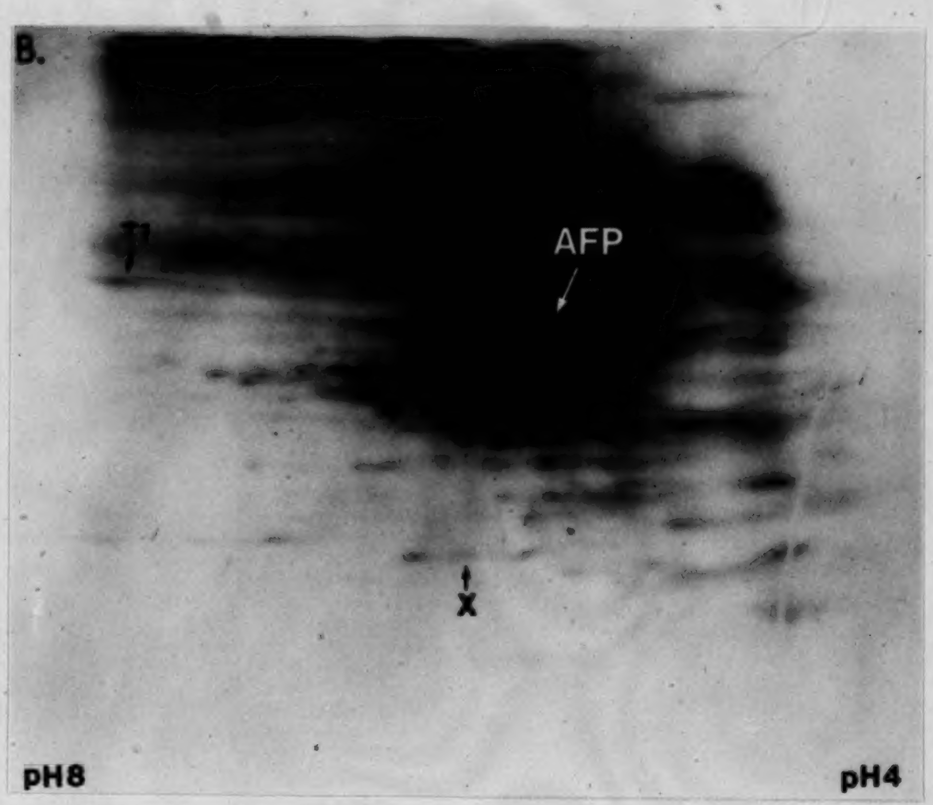
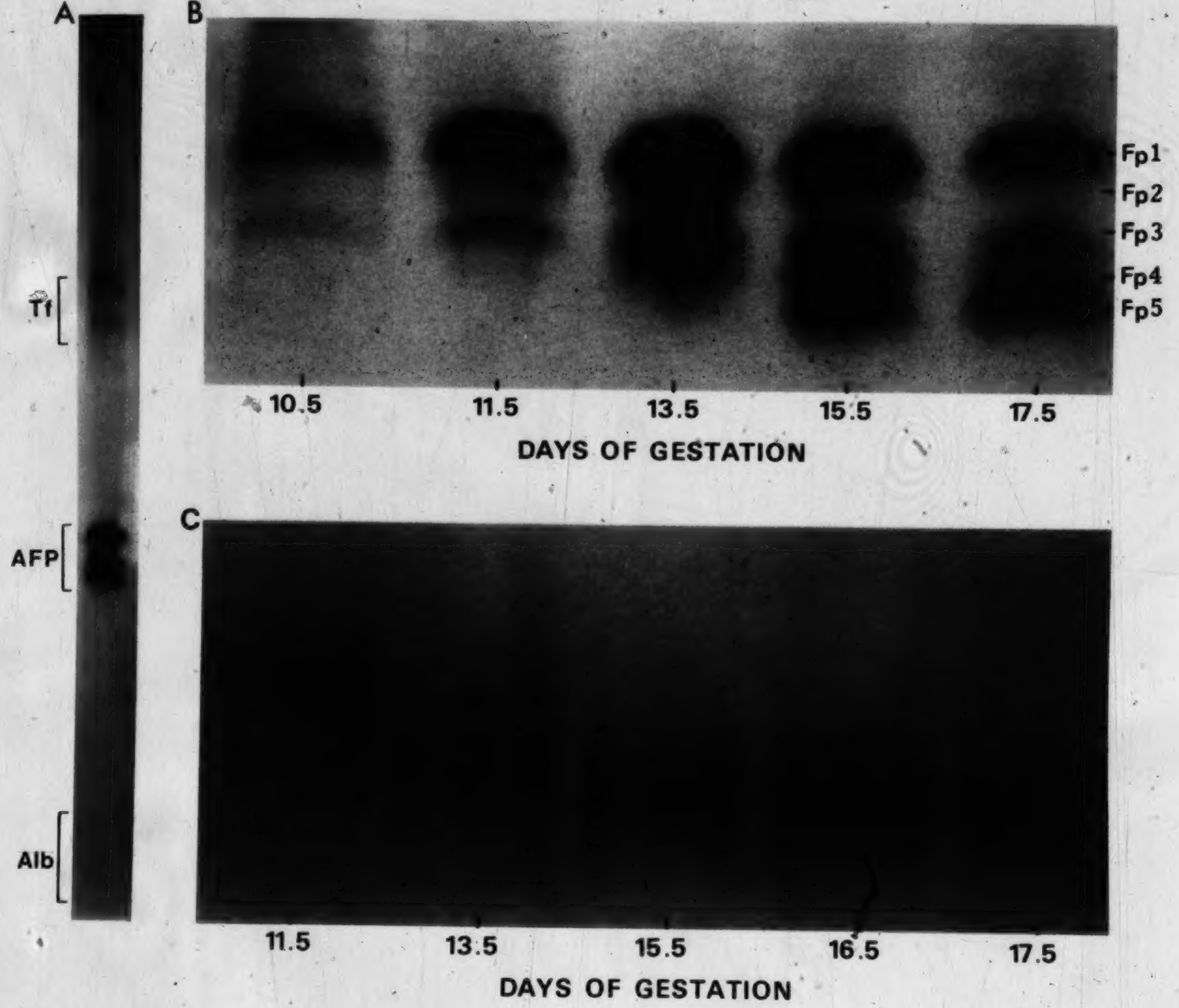

B.
T'
AFP
X
pH8
pH4

Figure 20. Developmental changes in the microheterogeneity of AFP analyzed by alkaline gel electrophoresis. Yolk sacs at different stages of gestation were cultured in medium containing [^{35}S] methionine. For A and B the yolk sacs were lysed in the culture medium as described in Materials and Methods. Equal counts from each day of gestation were applied to a 300 mm long by 1.5 mm thick alkaline gel. Electrophoresis, gel staining, and gel preparation for fluorography were carried out as described in Materials and Methods. (A) A fluorogram of the day 15.5 lysate is shown. The Tf, AFP and albumin regions are indicated. (B) A fluorogram of the AFP region from each of the days of gestation is shown. The bands are designated by the nomenclature of Zimmerman (241) where Fp 1 is the unsialylated form of AFP and Fp 2,3,4, and 5 contain increasing amounts of sialic acid. (C) The yolk sacs and medium were separated and only the secreted AFP analyzed. Approximately equal AFP counts were applied to each slot of an alkaline gel. Electrophoresis and treatment of the gel to visualize the radioactive bands were as described in Materials and Methods. The AFP region of the fluorogram is shown

then analyzed by two dimensional electrophoresis (Fig. 19B). Although the resultant fluorogram was rather streaky, AFP was clearly the major glycosylated protein. There were two intense spots in the high molecular weight region. While the remainder of the radioactive proteins were all of minor intensity, there were a large number of them. Tf was expected to be present but there was only a minor streak present in the Tf region. From the previous analysis of cellular proteins (Fig. 10), protein X did not appear to be glycosylated.

(b) Changes in glycosylation of AFP with gestation

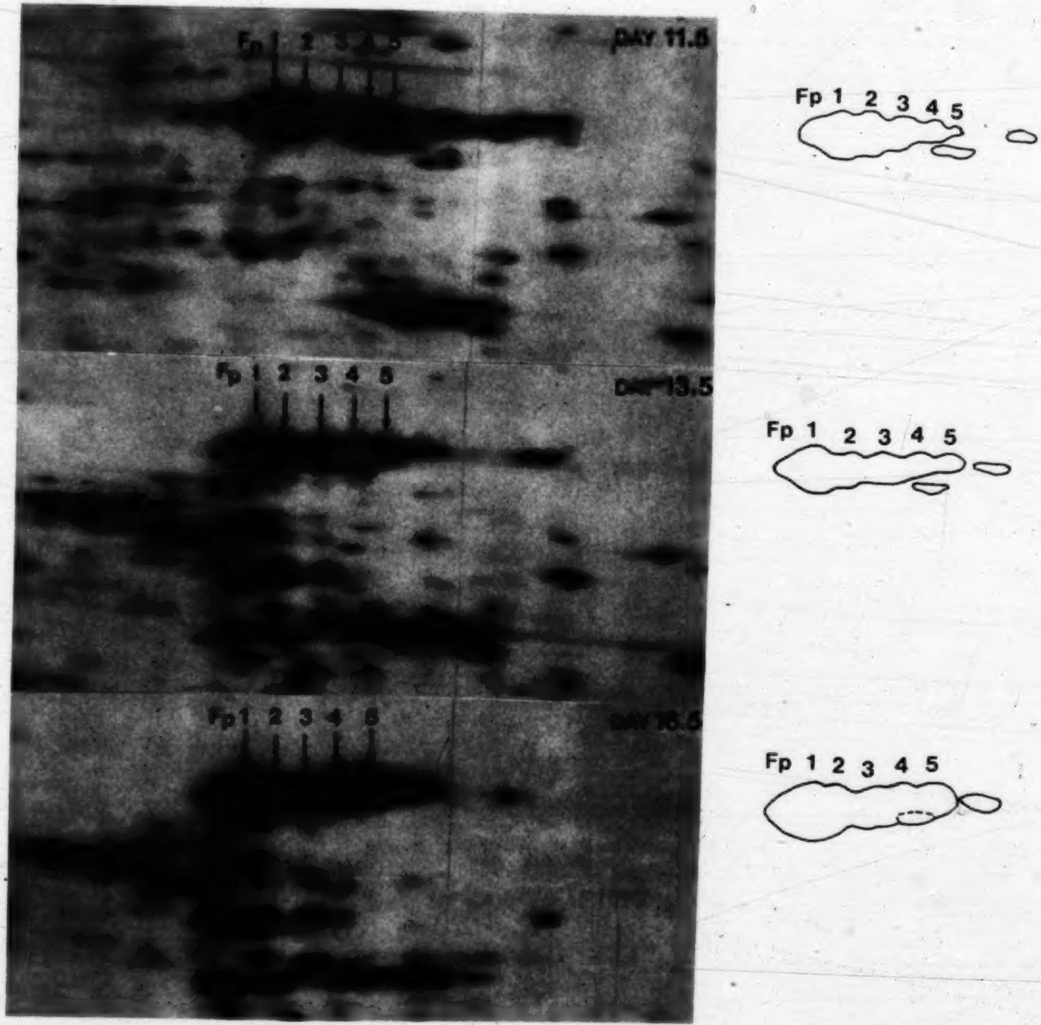
Previous studies in mice by Zimmerman (241) have shown that the sialylation of AFP changes during gestation and this causes the microheterogeneity seen in AFP. To detect the heterogeneity and its changes during gestation, two types of electrophoresis were used. Alkaline gel electrophoresis (231) has been traditionally used to separate serum proteins, but when long gels and increased resolving time were used, the different forms of Tf and AFP were separated (Fig. 20A). AFP was the major labelled protein and was resolved into a number of bands. There was also some activity present in the Tf region, but none in the albumin region. The AFP region of different gestational days showed that at each day the Fp-1 form was always one of the predominant bands, while the remainder of the forms changed during gestation (Fig. 20B). At days 10.5 and 11.5, most of the radioactivity not in Fp-1 was in Fp-3 with some possibly in Fp-2. At day 13.5, Fp-4 became significant and by day 15.5, Fp-5 was also present. By day 17.5, Fp-5 was an intense band with faint intensity at Fp-3 and Fp-4. Therefore, during gestation, there is a apparent shift of radioactivity in non-Fp-1


A
Tf
AFP
Alb
B
Fp1
Fp2
Fp3
Fp4
Fp5
10.5
11.5
13.5
15.5
17.5
DAYS OF GESTATION
C
11.5
13.5
15.5
16.5
17.5
DAYS OF GESTATION

AFP to more acidic forms. The bands in the secreted fractions were not very intense (Fig. 20C). They did however show the change in the forms of of AFP that were found in the medium. At day 11.5, the radioactivity was higher on the gel probably at the Fp-1 position, while at day 13.5 it shifted to a rather diffuse series of bands at a more acidic position. From day 15.5 to 17.5, the most acidic band became more intense. This was probably the Fp-5 form. A comparison with Fig. 20B revealed that no significant Fp-1 band was present from days 13.5 to 17.5. This indicates that Fp-1 is probably not secreted late in gestation and is the original cellular form which becomes sialylated during the secretion process, producing the other forms of AFP which are secreted.

Another method used to separate different charge forms was isoelectric focussing. The two dimensional system was used in an attempt to resolve these different species of AFP and detect gestational changes (Fig. 21). In each case, the surrounding spots interfered with clear visualization of AFP and the changes in its heterogeneity. In an attempt to clarify these changes, tracings of the AFP region were made and shown. The different forms were also designated with arrows. The results were the same as for the alkaline gel electrophoresis in that Fp-1 was always the major form and the remainder of the spots changed during gestation. At day 11.5, Fp-2 was the major spot with each of the other forms decreasing in intensity to spot Fp-5 which was very small. At day 13.5, the intensities of the spots had shifted so that Fp-2, 3 and 4 were approximately equal and Fp-5 also increased substantially. At day 16.5, Fp-2 and 3 decreased and Fp-4 and Fp-5 were the major forms. These results were similar to those in Fig. 20B in that the more acidic forms predominate towards the end of gestation.

Figure 21. Developmental changes in the microheterogeneity of AFP analyzed by two dimensional electrophoresis. Yolk sac lysates at different stages of gestation were prepared as in Figure 20A. Samples from day 11.5, 13.5 and 16.5 were analyzed by two dimensional gel electrophoresis. The radioactive protein spots were visualized by fluorography. The AFP region of the gels was photographed and tracings of the AFP spots were made in order to see the differences with gestational age. The nomenclature of Zimmerman (241) was used to denote the different forms of AFP where Fp 1 indicated the unsialylated form and Fp 2 - 5 are the sialylated forms of AFP. Two other proteins near the AFP region were also denoted.


Fp 1 2 3 4 5
DAY 11.5
Fp 1 2 3 4 5
Fp 1 2 3 4 5
DAY 13.5
Fp 1 2 3 4 5
Fp 1 2 3 4 5
DAY 15.5
Fp 1 2 3 4 5

The sialylated forms of AFP are probably the ones that are secreted while Fp-1 remains internal.

(c) Effect of tunicamycin on glycosylation of AFP

AFP is glycosylated and secreted by the yolk sac. Tunicamycin inhibits the N-linked glycosylation of protein, by interfering with the attachment of carbohydrate to asparagine. In these experiments, the effect of tunicamycin on the synthesis and secretion of AFP was studied. The synthesis and glycosylation of total protein and AFP were studied by the incorporation of [^{35}S] methionine into the protein (Table 3A) and of [^{3}H] glucosamine into the carbohydrate portion of glycoproteins (Table 3B). There was a 21% decrease in protein synthesis at 2 hr of treatment which increased to 36% at 6 hr. The antibiotic did not seem to have any effect on the relative rates of AFP synthesis, indicating that the inhibition of protein synthesis was a general effect. The glycosylation of cellular protein was inhibited by 73% at 2 hr and 89% at 6 hr. The relative rate of AFP glycosylation was reduced by 60% at 2 hr and by 86% at 6 hr. These results indicate tunicamycin causes a small inhibition of protein synthesis and a dramatic inhibition of protein glycosylation which seems to specifically inhibit AFP glycosylation.

Tunicamycin treatment reduced total protein secretion by 35% at 2 hr and 46% at 6 hr (Table 4A). These values were similar to the reduction in total protein synthesis (Table 3A). There was a small reduction in the relative secretion of AFP at 2 hr but no apparent effect at 6 hr. The reduction at 2 hr could be due to an overestimation of the relative secretion of AFP in the control at 2 hr (62%). There was a reduction in the glycosylation of secreted proteins

Table 3. Effect of tunicamycin on the synthesis and glycosylation of yolk sac cellular protein

Yolk sacs from day 15.5 of gestation were cultured for the indicated times, then separated from the medium and lysed. The treated cultures had tunicamycin present throughout the incubation period, while the isotopes [^{35}S] methionine (in A) and [^{3}H] glucosamine (in B) were present only during the last 1.5 hr of the incubation in both control and treated samples. The lysates were analyzed for total protein, total isotope incorporation, and AFP synthesis and glycosylation, the latter of which were determined by immunoprecipitation.

A.

Incubation Time (hr)	INCORPORATION OF [^{35}S] METHIONINE					
	Control		Treated		% of Control	
	Total (cpm/mg protein)	AFP (% of total)	Total (cpm/mg protein)	AFP (% of total)	Total	AFP
2	2.35×10^6	16.3	1.85×10^6	19.0	79	116
6	4.30×10^6	18.8	2.77×10^6	17.7	64	94

B.

Incubation Time (hr)	INCORPORATION OF [^{3}H] GLUCOSAMINE					
	Control		Treated		% of Control	
	Total (cpm/mg protein)	AFP (% of total)	Total (cpm/mg protein)	AFP (% of total)	Total	AFP
2	94,300	15.4	25,500	6.2	27	40
6	63,300	19.4	7,000	2.9	11	14

Table 4. Effect of tunicamycin on the secretion of total and glycosylated protein by the yolk sac

The secreted proteins found in the medium of cultures described in Table 3 were analyzed for radioactivity in total protein and in AFP immunoprecipitates. (A) [^{35}S] methionine-labelled proteins. (B) [^{3}H] glucosamine-labelled proteins.

A:

Incubation Time (hr)	PROTEINS SECRETED				% of Control	
	Control		Treated			
	Total (cpm/100ul medium)	AFP (% of total)	Total (cpm/100ul medium)	AFP (% of total)	Total	AFP
2	108,000	62	70,000	48	65	77
6	144,000	50	78,000	51	54	102

B.

Incubation Time (hr)	GLYCOSYLATED PROTEIN SECRETED				% of Control	
	Control		Treated			
	Total (cpm/100ul medium)	AFP (% of total)	Total (cpm/100ul medium)	AFP (% of total)	Total	AFP
2	9000	40	5000	22	56	55
6	7400	30	1000	10	14	33

by 44% at 2 hr and 86% at 6 hr (Table 4B). The amount of glycosylated AFP in the secreted fraction was reduced by 45% at 2 hr and by 67% at 6 hr. Tunicamycin inhibits the glycosylation of AFP but does not affect the AFP secretion.

Since tunicamycin inhibits the glycosylation of AFP, the molecular weight and the heterogeneity of AFP should change. The effect of tunicamycin on the molecular weight of AFP from secreted and cellular fractions and on the total secreted fraction are shown (Fig. 22). The minor bands of the total secreted protein (Fig. 22A, slot 1 and 2) did not change; however, there were two major bands which showed small but distinct changes in molecular weight. Such changes in molecular weight were found in the AFP immunoprecipitates of the secreted fraction (slot 3 and 4). AFP was the major component of the secreted fraction and Tf was just above AFP. These results indicate that Tf also changes in molecular weight during tunicamycin treatment. The immunoprecipitate of the cellular fraction (Fig. 22B) showed the molecular weight of AFP in yolk sac cells was reduced by tunicamycin treatment. This indicates that the inhibition of glycosylation by tunicamycin is taking place in the cells. Although the change in molecular weight was difficult to quantitate in this region of the gel, the average change in four determinations was 4.2%. Previously published values found the amount of carbohydrates on AFP to be 4%. These results also indicate that non-glycosylated AFP is secreted.

This point is best demonstrated by a change on two dimensional gels (Fig. 23). There was a striking difference in the heterogeneity from the control to the treated samples. In the treated sample, there was one major spot present, while in the control the typical series of four

Figure 22. Analysis by SDS gel electrophoresis of AFP synthesized and secreted by tunicamycin treated yolk sacs. Yolk sacs were incubated in the presence or absence of tunicamycin (2 μg/ml) for 6 hr. [^{35}S] methionine was present during the last 1.5 hr of incubation. Secreted and cellular protein fractions were prepared and the immunoprecipitation of each fraction was carried out as described in Materials and Methods. (A) Total secreted protein and secreted AFP from control and treated cultures were analyzed on SDS 10% polyacrylamide gels. (B) Immunoprecipitated AFP from the cellular fraction was analyzed on the same slab gel.

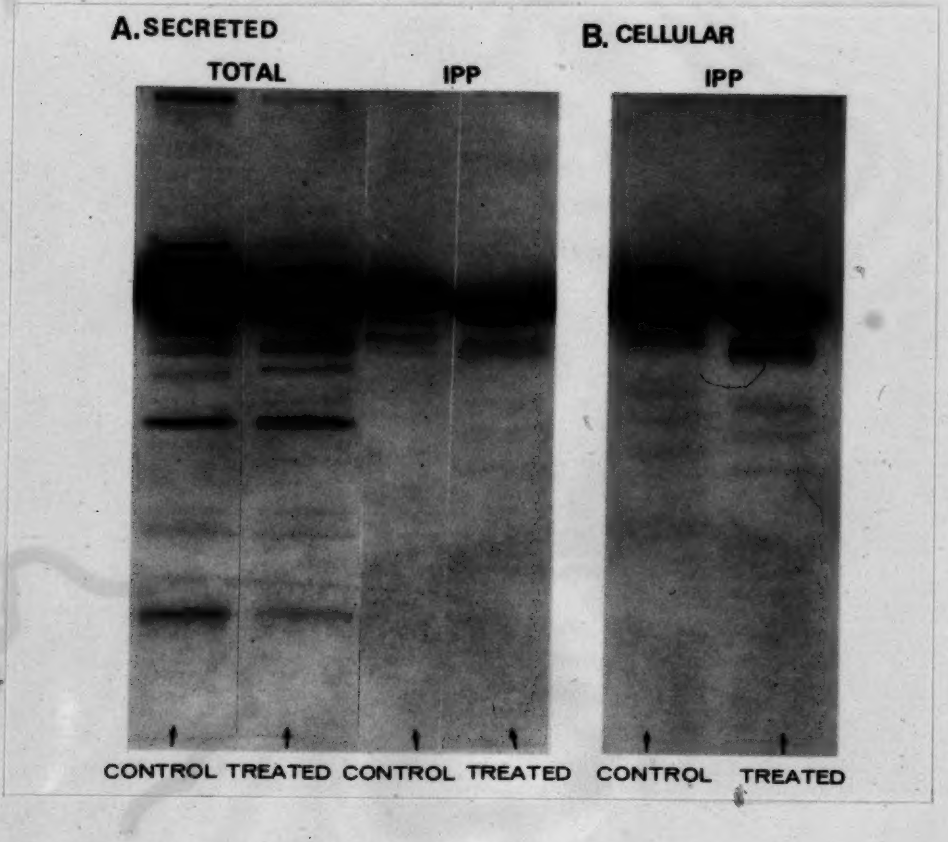
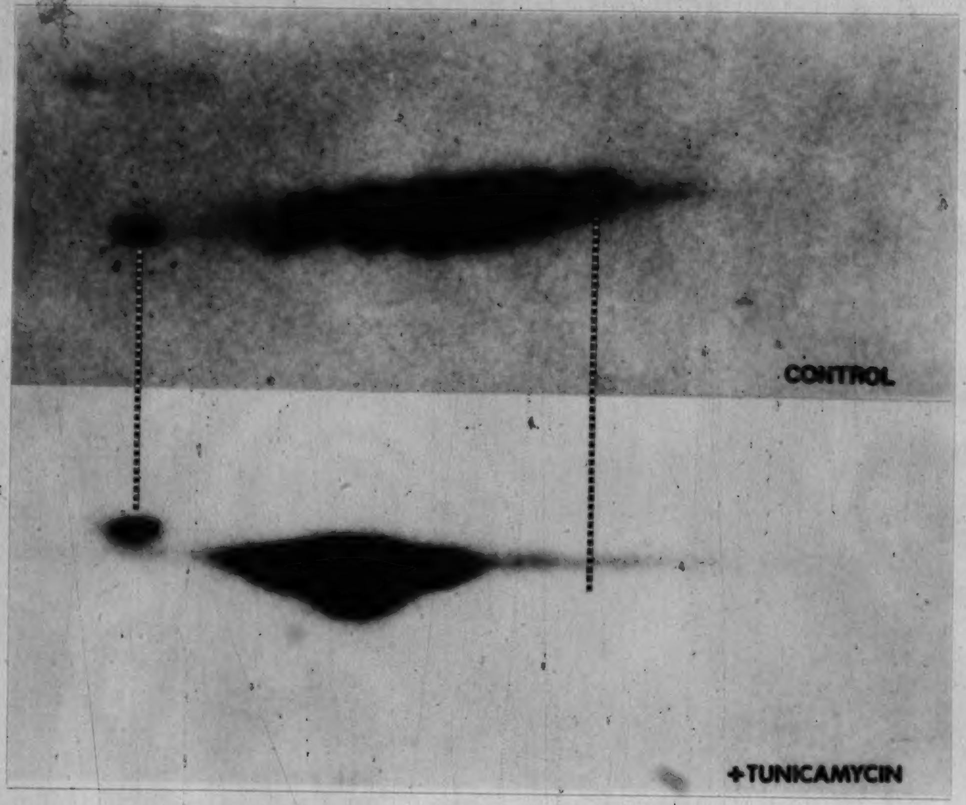

A. SECRETED
TOTAL
IPP
B. CELLULAR
IPP
CONTROL
TREATED
CONTROL
TREATED
CONTROL
TREATED

Figure 23. Two dimensional analysis of AFP secreted by tunicamycin treated yolk sacs. Total secreted protein from samples described in Figure 22 were analyzed by two dimensional gel electrophoresis. Enlargements of the AFP regions of the fluorograms for control and treated samples are shown. Using the non-AFP protein on the left as a marker, the vertical lines drawn on each side denote the difference in microheterogeneity of the samples.


CONTROL
+TUNICAMYCIN

spots was present. The glycosylation of AFP was inhibited by tunicamycin, which resulted in a reduction of the heterogeneity. When the molecular weight of the treated and control AFP were compared to the minor protein to the left of AFP, there was an obvious decrease in treated sample. These results indicate that tunicamycin inhibits the glycosylation of AFP which decreased the molecular weight and the molecular heterogeneity of AFP.

C. Discussion

Immunoprecipitation analysis showed that the synthesis of AFP relative to total protein synthesis by the yolk sac increases during gestation, reaching a maximum at day 15.5, after which it decreases. This confirms the electrophoretic results in Chapter III (Fig. 9 and 10). Previous studies have shown that the relative proportion of AFP mRNA in the polysomes of the yolk sac increases to a maximum at day 14.5 and then decreases; day 15.5 or 16.5 were not analyzed (147). Therefore a good correlation exists between the changes in the relative amount of AFP mRNA and the changes in relative synthesis of AFP during gestation. This indicates that AFP synthesis is probably regluated by the amount of functional AFP mRNA.

During the latter half of gestation, AFP constitutes the major fraction of the secreted protein. This suggests that AFP may have some important function, either in the fetus or in the maternal-fetal relationship. The organelles of secretion (E.R. and Golgi apparatus) are located in the basal part of the cell, indicating that most of the secretion is into the intercellular space. Therefore AFP would first enter the fetal circulation and then eventually cross the placenta to enter the maternal serum.

In the present study, I found a time lag of 30 min before the appearance of AFP in the medium as compared to only 20 min for release of albumin from hepatoma cells (9). This difference may be due to the hepatoma cells secreting albumin directly into the medium while AFP release from the yolk sac is more complex due to its release into the intercellular space.

The changes in microheterogeneity of AFP during gestation agree with the work of Zimmerman (241). The more sialylated forms of AFP are usually secreted, while the unsialylated (Fp-1) is usually intracellular.

The secretion of AFP by the yolk sac presents itself as a model system to study the molecular mechanisms of protein secretion. Previous studies have shown that AFP is made initially as a preprotein containing a signal sequence of 20 amino acids which is removed during the secretion process (161). In these studies, the effect of glycosylation upon AFP secretion was investigated. Tunicamycin had a significant effect on the molecular weight and glycosylation of AFP, indicating that the presence of the carbohydrate portion was greatly reduced. This confirms that the main attachment site for carbohydrate to AFP is to asparagine. Tunicamycin had little or no effect on the secretion of AFP, suggesting that N-linked glycosylation of AFP is not required for its secretion. Similar conclusions have been found for the secretion of other glycoproteins, such as transferrin and VLDL (148, 201). This has led to the postulation that glycosylation might be important in protein structure or function but not for secretion.

CHAPTER V

CONTROL OF AFP GENE EXPRESSION

A. Introduction

The relative rate of AFP synthesis increases during gestation to a maximum at day 15.5. The level of synthesis appears to be regulated by the proportion of AFP mRNA in the polysomes (147). The amount of any mRNA present in the cytoplasm is the consequence of a steady state between its rate of synthesis and its rate of degradation. A change in either rate will result in changes in the level of mRNA. The rate of synthesis of mature message is dependent mainly on the rate of transcription of that gene although the rate of processing may also be important. The rate at which mRNA's are degraded varies with the mRNA This rate affects their relative abundance in the cytoplasm, which in turn determines the level of protein synthesis. The factors which control mRNA stability are poorly understood but may involve RNA structure and/or protein bound to the mRNA.

Since many differentiated proteins have very stable mRNA's, the stability of AFP mRNA was determined. mRNA stability has been studied in several ways. The earliest methods involved inhibiting the transcription of new mRNA and then assaying the decay of the mRNA by the decrease in protein synthesis (130, 154). Initially, actinomycin D was used as it inhibits DNA dependent RNA synthesis. Using this inhibitor, Schimke showed that during the inhibition, the synthesis of some proteins was higher than that of others (154). These mRNA's were more stable than the remaining population and the effect was called superinduction.

Actinomycin D is no longer used for mRNA half life determinations as it has side effects which may cause artifacts (127). In the experiments reported here, the inhibitor α-amanitin was used. At low concentrations of α-amanitin, RNA polymerase II is inhibited,reducing the synthesis of mRNA. This then should reduce protein synthesis in a similar manner to actinomycin D.

A more direct approach to measure the decay of RNA has been developed which entails the radioactive labelling of a population of RNA's and then measuring the decay of this population (86). This method is more natural in the sense that all normal functions are maintained in the cells during the experiment. In this procedure, the cells are labelled for a short period of time with [^{3}H] uridine (pulse) and then uridine, cytidine and glucosamine are added to dilute out the label from the UTP pool and to stop further incorporation into RNA (chase). The cessation of isotope incorporation must be complete to obtain an accurate determination of the RNA decay.

The measurement of a specific mRNA in a complex population has now been greatly simplified by advances in molecular cloning. This technology allows the amplification of DNA sequences complementary to specific mRNA's which can be used as selective probes in hybridization assays. To assay the decay of AFP mRNA in the yolk sac, total RNA was pulse labelled, chased for various lengths of time and radioactivity in AFP mRNA was assayed by hybridization with DNA sequences specific for AFP mRNA. The stability of AFP mRNA was determined from the decay curve.

B. Results

(1) Transcription in the Yolk Sac

(a) Effect of α-amanitin on AFP synthesis

The effects of α-amanitin on total protein synthesis and AFP synthesis are shown (Fig. 24A). Total protein synthesis was reduced by 50% at 7.5 hr and by 60% at 25.5 hr. During this incubation period, there was no apparent decrease in AFP synthesis. This resulted in an increase in the relative rate of AFP synthesis for the treated sample, from 20% to nearly 40% at 25.5 hr of culture (Fig. 24B). The relative synthesis of AFP in the control cultures was constant at 17-19% of total protein synthesis during the incubation period.

(b) Effect of α-amanitin on total protein synthesis

Total labelled proteins were analyzed by two dimensional gel electrophoresis to compare the protein synthesized by control and α-amanitin treated yolk sac (Fig. 25). In general comparison, the most obvious difference was a reduction in the background radioactivity on the gels of treated yolk sacs, indicating that the synthesis of many minor proteins had decreased. The protein spots that remained after treatment became more distinct. There were several examples of proteins with intermediate intensity that disappeared completely during treatment and these have been designated with upward arrows (Fig. 25). The main example was protein Y, whose intensity was greatly reduced by inhibitor treatment. The proteins AFP, Tf, X and Y' constituted the major portion of the protein synthesized by the yolk sac at this stage. There seemed to be little change in their relative intensities after treatment. There were many spots of intermediate intensity which were still present

Figure 24. Effects of α-amanitin on AFP synthesis in yolk sac explants

Yolk sacs at day 15.5 of gestation were cultured for 1.5, 7.5 and 25.5 hr. [^{35}S] methionine was added during the last hour of incubation. The treated cultures had α-amanitin present throughout the incubation period at a concentration of 10 µg/ml. Lysates were prepared from the yolk sacs and analyzed for protein content, hot TCA precipitable radioactivity and AFP immunoprecipitable radioactivity. (A) The specific activities of total protein and of AFP synthesized by the treated samples were plotted as a function of incubation time. (B) The relative rate of AFP synthesis in the treated cultures was compared directly to that of control samples during the incubation period.

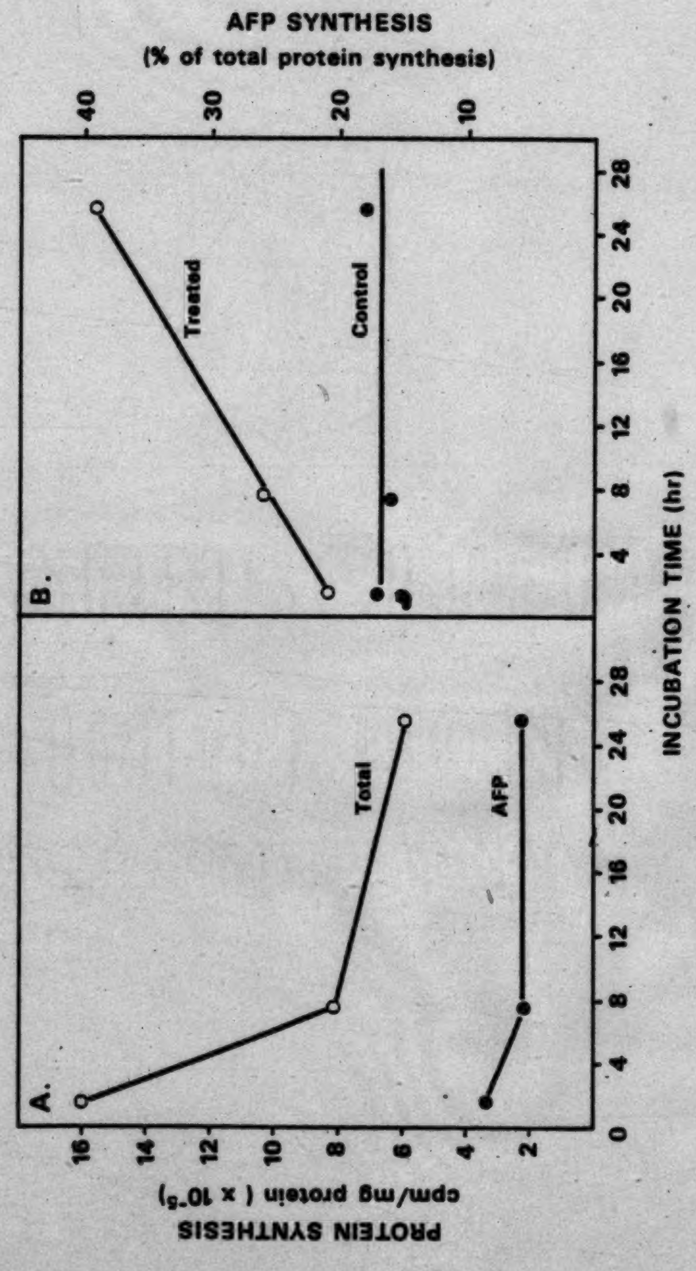
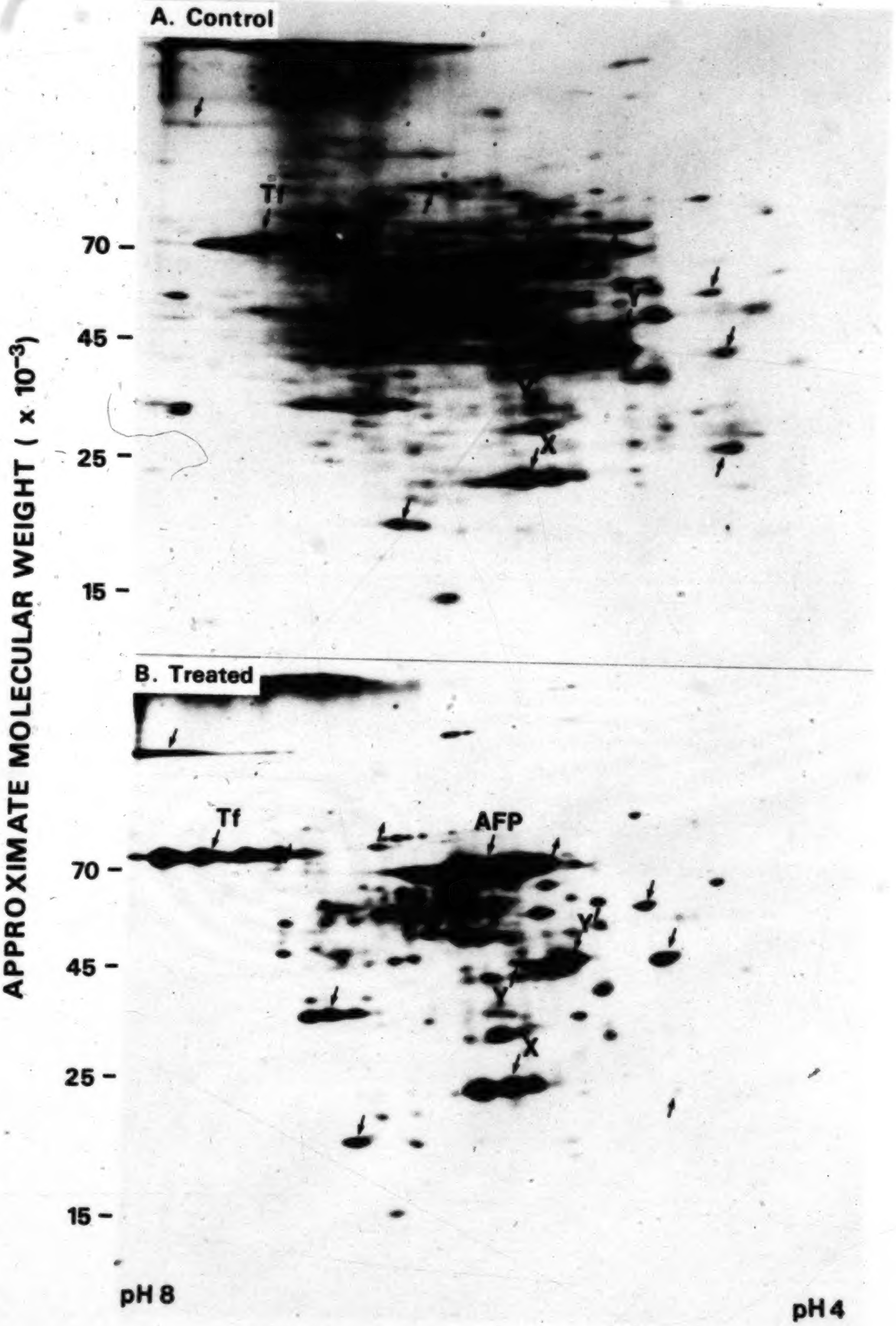

A.
B.
PROTEIN SYNTHESIS
cpm/mg protein (x 10^-5)
16
14
12
10
8
6
4
2
0
Total
AFP
Treated
Control
AFP SYNTHESIS
(% of total protein synthesis)
40
30
20
10
4
8
12
16
20
24
28
INCUBATION TIME (hr)

Figure 25. Effects of α-amanitin on protein synthesis in yolk sac explants. Yolk sacs were incubated with or without α-amanitin for 25.5 hr as described in the legend of Figure 24 and lysed. Proteins (200,000 cpm) from the control and treated lysates were analyzed by two dimensional electrophoresis. Labelled proteins were visualized by fluorography. The previously identified spots are labelled as Figure 8. Downward arrows (↓) indicate spots which remain after treatment, while upward arrows (↑) are representative of those proteins which disappear.


A. Control
Tf
Y
X
B. Treated
Tf
AFP
Y
X
70 –
45 –
25 –
15 –
APPROXIMATE MOLECULAR WEIGHT (x 10⁻³)
pH 8
pH 4

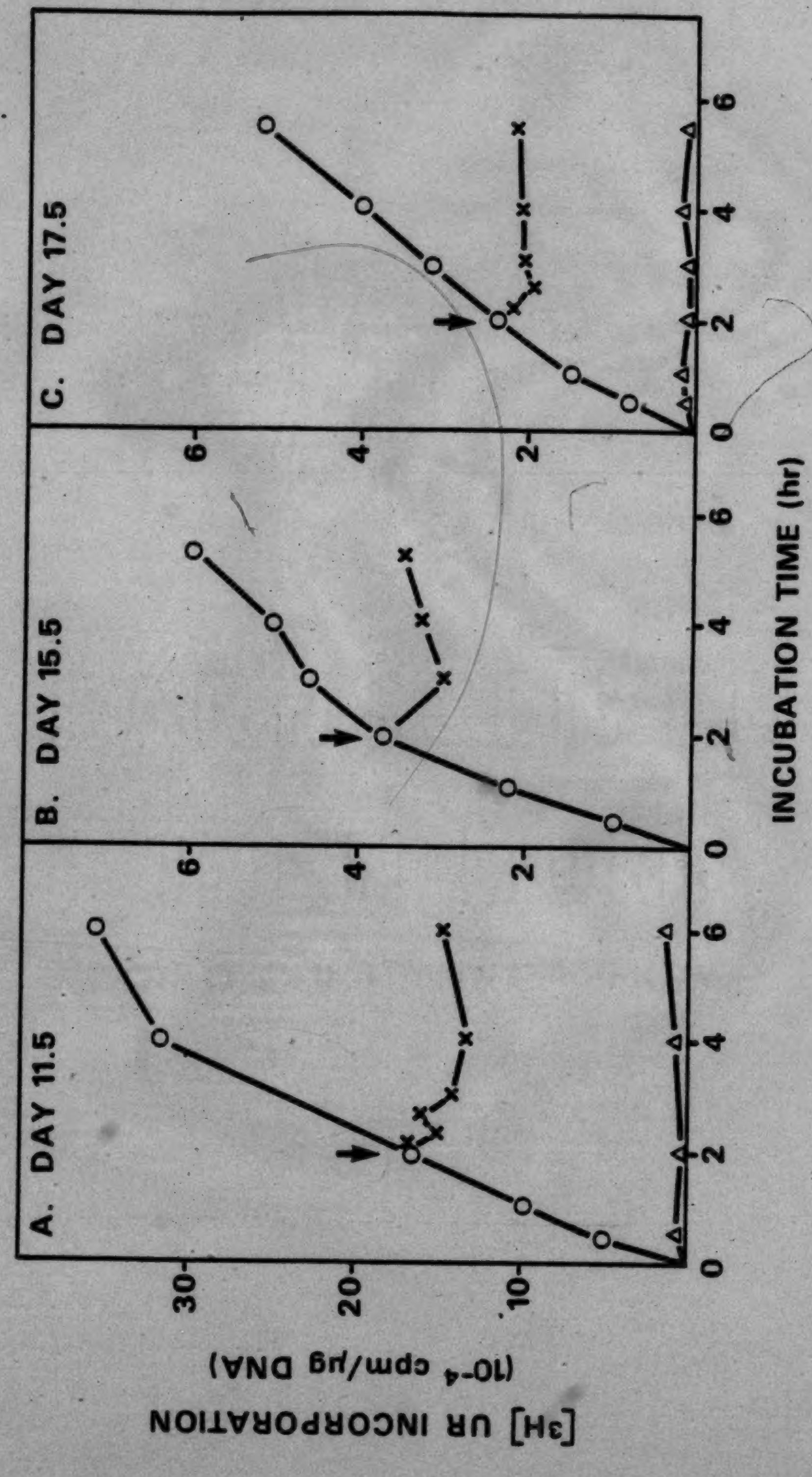
after treatment and examples of these are indicated in Fig. 25 by downward arrows. In some cases, these proteins actually showed small increases in intensity. The inhibitor had no apparent effect on the major proteins synthesized in the yolk sac at this stage of gestation.

(2) Stability of AFP mRNA During Gestation

(a) Total mRNA

To study the stability of various RNA species by pulse and chase methodology, certain experimental conditions had to be determined. First, all RNA species have to be labelled to a high specific activity. This was especially true for the determination of the half life of AFP mRNA. Secondly, the chase must cause a rapid cessation in isotope incorporation so that true decay behaviour is displayed. For each age tested, the incorporation of [^{3}H] uridine (0.25 mCi/ml) increased linearly to 5-6 hr of culture (Fig. 26). For the following experiments, an incubation period of 4 hr was chosen. Both mRNA and rRNA were labelled under these conditions (see Chapter III). The chase was initiated by removing the radioisotope containing medium and replacing it with several fold excess of medium containing 5 mM uridine, 5 mM cytidine and 5 mM glucosamine. These conditions have been shown to reduce the [^{3}H] uridine available for RNA synthesis (122). The results showed that for day 11.5 and 17.5 there was a cessation in uridine incorporation within 10 min of adding the chase medium and no further incorporation took place after 10 min. The [^{3}H] uridine labelled material was alkali labile, insuring that incorporation was into RNA.

Figure 26. Kinetics of [^{3}H] uridine incorporation into yolk sac RNA under pulse and chase conditions. Yolk sacs at day 11.5, 15.5 and 17.5 of gestation were cultured in medium containing [^{3}H] uridine for indicated times. Incorporation of [^{3}H] uridine into RNA was analyzed by TCA precipitation of yolk sac lysates (O—O) as a function of incubation time. For day 11.5 and 17.5, samples at each time point were treated with 0.5 N NaOH for 30 min at 60°C before TCA precipitation (Δ—Δ). After labelling the yolk sac for 2 hr, the medium was changed to 1.5-3.0 volumes of chase medium (indicated by the arrow) and samples taken at indicated times (X—X).


A. DAY 11.5
B. DAY 15.5
C. DAY 17.5
[3H] UR INCORPORATION
(10-4 cpm/µg DNA)
30
20
10
6
4
2
0
2
4
6
INCUBATION TIME (hr)

The RNA was isolated from the yolk sacs during the chase and the decay of total RNA and poly $(A)^+$ RNA was determined (Fig. 27). The decay of the total RNA was slow compared to poly $(A)^+$ RNA and represented essentially the decay of rRNA as it constitutes greater than 95% of the total RNA. The decay of total RNA was linear with a half life of 52 hr. The stability of the total RNA did not change much during gestation. The poly $(A)^+$ RNA of day 11.5 and 15.5 yolk sacs both had an initial half life of 4-6 hr, while after 12-20 hr of chase, there was an apparent shift to a slower decay. Day 17.5 yolk sac poly $(A)^+$ RNA had a single component decay with a half life of 10-15 hr.

(b) AFP mRNA

To specifically follow the decay of AFP mRNA, plasmid pAF-7 which contained DNA sequences complementary to 42% of the AFP mRNA was bound to nitrocellulose filters and $[^3H]$ uridine labelled RNA was hybridized to it (Fig. 28). There was a linear relationship between bound radioactivity and the amount of $[^3H]$ RNA added (data not shown). The hybridization conditions to assay the decay of AFP mRNA of each gestational day were identical and therefore the percent bound radioactivity could be plotted directly. It appeared that AFP mRNA has a very long half life as there was no apparent decay by 30 hr of chase. There was a large scatter in the points and this made it difficult to calculate an exact half life. In the case of day 11.5, AFP mRNA decay was also measured using the poly $(A)^+$ fraction; AFP mRNA showed a long half life, but the scatter appeared to be reduced. These results demonstrated that this mRNA species was preferentially stabilized in the yolk sac and the half life of AFP mRNA did not appear to change during gestation.

Figure 27. Decay kinetics of total and poly (A)$^+$ RNA in the yolk sac explants obtained from different gestational stages. Yolk sacs at different days of gestation were incubated in medium containing $[5,6\text{-}^{3}H]$ uridine for 4 hr, at which time some of the yolk sacs were frozen. These were the zero time samples. The remainder of the yolk sacs were placed into chase medium and incubated further. Samples were taken at indicated times and frozen. RNA was extracted from the samples by the SDS-phenol method. The specific activity of the total RNA was determined by measuring the concentration and radioactivity as described in Materials and Methods and plotted as a percent of the maximum value (●–●), which was usually the zero time point. The total RNA was fractionated into poly (A)$^+$ and poly (A)$^-$ fractions by oligo d(T) cellulose chromatography and the proportion of the retained radioactivity to the total radioactivity applied was determined. This value was plotted as a percent of the zero time value (X–X).

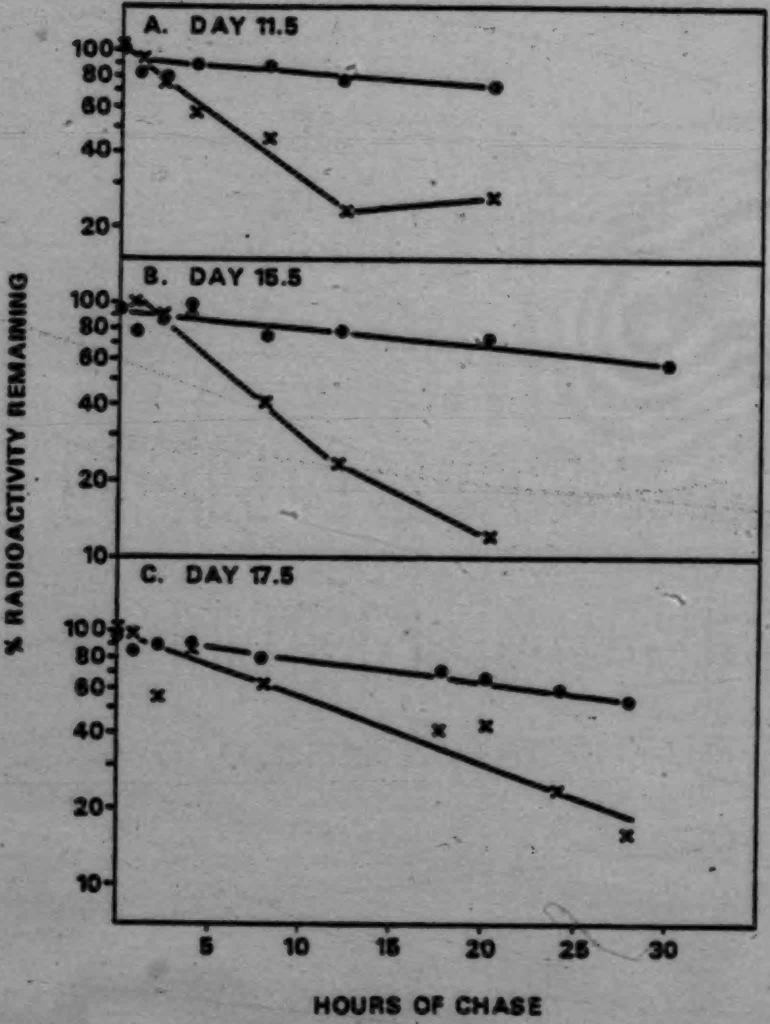
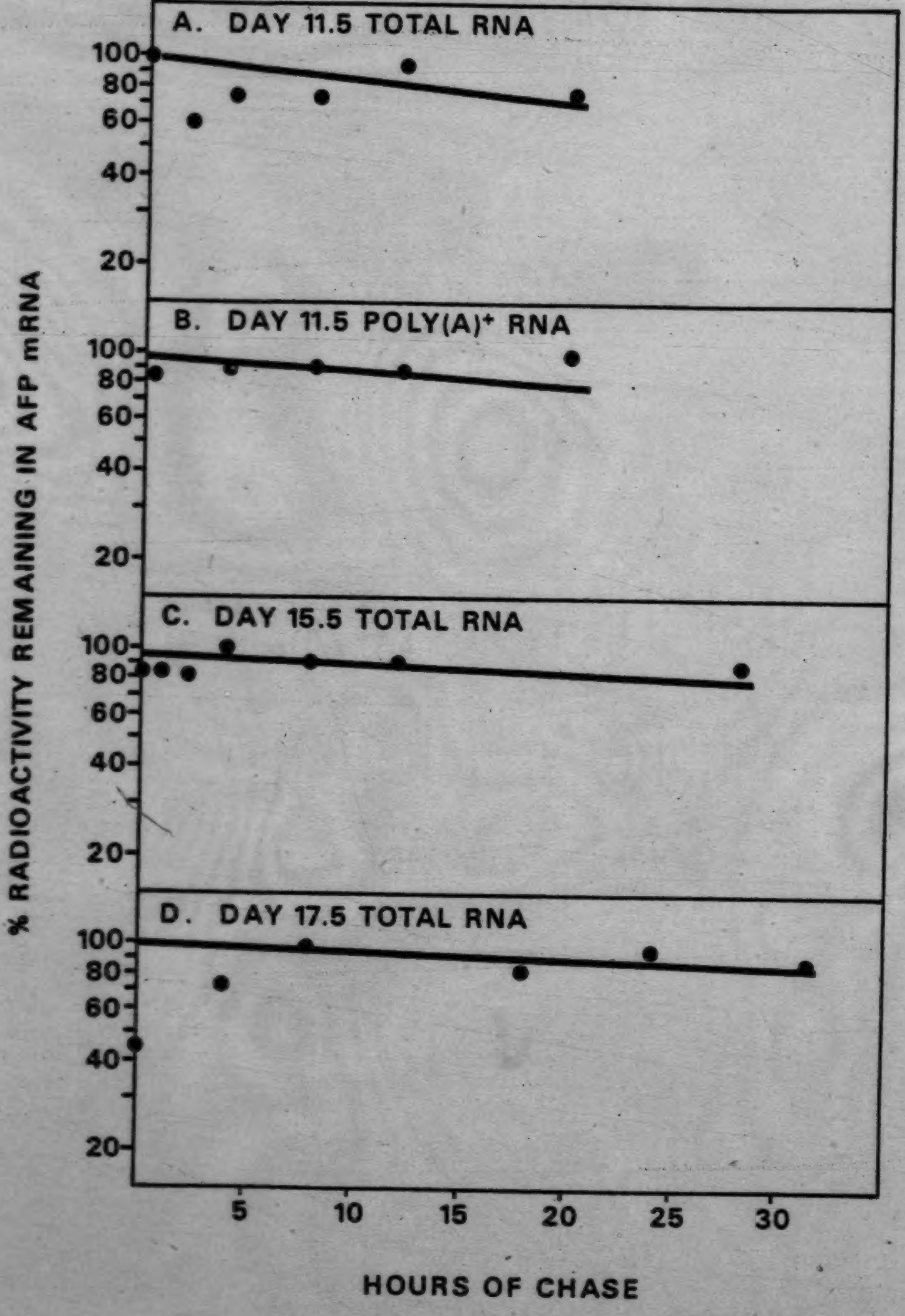

A. DAY 11.5
B. DAY 15.5
C. DAY 17.5
% RADIOACTIVITY REMAINING
100
80
60
40
20
10
5
10
15
20
25
30
HOURS OF CHASE

Figure 28. Decay kinetics of AFP mRNA in yolk sac explants obtained from various stages of gestation. The plasmid pAF-7 was immobilized on nitrocellulose membranes and labelled RNA prepared as described in Figure 27, was hybridized under conditions described in Materials and Methods. In part A, day 11.5 yolk total RNA, 64 μg RNA containing 3-8 x 10^6 cpm were added to each reaction. In part C, day 15.5 total RNA, 77 μg with 6-10 x 10^5 cpm, and in part D, day 17.5 total RNA, 159 μg with 1.5-2.5 x 10^5 cpm were hybridized to filter bound DNA. In part B, the poly $(A)^+$ RNA isolated from day 11.5 total RNA had 0.7 to 4.7 x 10^5 cpm added to each hybridization reaction. Duplicate samples were hybridized for each time point. The cpm bound per μg RNA hybridized was determined and the results were expressed as a percent of the maximum bound value.


A. DAY 11.5 TOTAL RNA
B. DAY 11.5 POLY(A)⁺ RNA
C. DAY 15.5 TOTAL RNA
D. DAY 17.5 TOTAL RNA
100
80
60
40
20
% RADIOACTIVITY REMAINING IN AFP mRNA
5
10
15
20
25
30
HOURS OF CHASE

The half life of the various RNA fractions were tabulated for the different days of gestation (Table 5). In the case of total RNA, the best line was extrapolated to 50% decrease and the value taken from the graph. At day 11.5 and 15.5 there was apparently no change in the half life of total RNA but by day 17.5 there appeared to be a 33% decrease. The variation in the points for total RNA decay was small, so this change could represent a real difference. The rapidly decaying component of the poly $(A)^+$ RNA for day 11.5 and 15.5 passed through 50%, so the half life could be determined directly. The decay of the second component of the poly $(A)^+$ was not measured. The scatter in the points for day 17.5 poly $(A)^+$ RNA decay was large; therefore a best line was determined mathematically and a half life calculated. The half life of the day 17.5 mRNA population was at least double that of the earlier days. For the determination of the half life of AFP mRNA, lines were drawn which best represented the decrease in filter bound radioactivity and these lines were extrapolated to 50%. Due to the large variation in these points, it was difficult to determine if there was any significant difference between the half lives at different days of gestation. These values suggested that the half life of AFP mRNA in the yolk sac was greater than three days.

To ensure the specificity of the filter hybridization assay, various control experiments were done (Table 6). In these experiments, samples were bound to plasmid bound filters, a blank filter and a chicken DNA containing filter (Table 6A). The radioactivity bound to the blank and chicken DNA was essentially background. The plasmid containing

Table 5. The half life of yolk sac RNA during gestation

DAY OF GESTATION	HALF LIFE (hr)		
	SAMPLE		
	TOTAL RNA(1)	POLY (A)$^+$ RNA(1)	AFP mRNA(2)
11.5	54	5.4	80 (total RNA) 140 (poly (A)$^+$ RNA)
15.5	56	5.3	110 (total RNA)
17.5	37	11.5	150 (total RNA)

Values for the half life of the various RNA populations in the yolk sac during gestation were determined from Figures 27 and 28.

(1) from figure 27
(2) from figure 28

Table 6. Characteristics of the filter hybridization assay.

(A) Total day 11.5 [^{3}H] RNA was hybridized to plasmid bound filters or control filters (blank and chicken DNA) under conditions described in Materials and Methods.

(B) Total [^{3}H] RNA (147,000 cpm/µg) isolated from Chinese hamster cells (DON) (gift from B. Biederman, University of Calgary), was hybridized to plasmid bound filters to estimate background binding. The plasmid pAF-6 contained DNA sequences complementary to 75% of the AFP mRNA. (C) To estimate the efficiency of binding, [^{3}H] cDNA (1.5×10^6 cpm/µg) made from pure AFP mRNA using reverse transcriptase (gift from G. Andrews, University of Calgary) was hybridized to plasmid bound filters. After correcting for the differences in counting efficiency between liquid and filter bound radioactivity, the efficiency of cDNA binding was 13.5%.

DNA ON FILTER	RNA	INPUT cpm	BOUND cpm
A. BLANK	$[^3H]$ TOTAL DAY 11.5 YOLK SAC	0.9×10^6	18
CHICKEN	$[^3H]$ TOTAL DAY 11.5 YOLK SAC	1.0×10^6	23
pAF-7	$[^3H]$ TOTAL DAY 11.5 YOLK SAC	0.9×10^6	296
pAF-7	$[^3H]$ TOTAL DAY 11.5 YOLK SAC	1.0×10^6	275
B. pAF-6	$[^3H]$ DON CELL TOTAL	1.5×10^6	26
BLANK	$[^3H]$ DON CELL TOTAL	1.5×10^6	21
C. pAF-6	$[^3H]$ cDNA_{AFP}	17,000	994
BLANK	$[^3H]$ cDNA_{AFP}	17,000	54

filters bound radioactive RNA 10 fold over the control filters. In all experiments, the bound activity was calculated by subtracting the background activity. The background binding of radioactive RNA to the filters was determined using an RNA which theoretically has no sequences complementary to mouse AFP (Table 6B). The counts bound to both the blank and plasmid containing filter were essentially at background levels. cDNA made from AFP mRNA was hybridized to provide an estimate for the efficiency of the binding assay (Table 6C). The efficiency was calculated to be 13.5%. These results indicated that the radioactive yolk sac RNA bound to the plasmid insert specific for AFP mRNA.

C. Discussion

α-Amanitin had a profound effect on protein synthesis in the yolk sac explants. The analysis of the protein synthesis in the α-amanitin treated sample showed the presence of many major proteins and a lack of many minor ones. The proteins which are probably required for specialized functions seem to have more stable mRNA's than the minor "housekeeping" proteins. The proteins which appeared to be coordinately expressed (AFP, Tf, X and Y') apparently have mRNA's with long half lives. During the course of α-amanitin treatment, AFP synthesis did not appear to change and this led to a 2 fold increase in the synthesis of AFP relative to that of other proteins. This indicated that the half life of AFP mRNA is greater than most other yolk sac mRNA's and was greater than 25 hr.

The pulse and chase methodology was used to determine the half life of the total RNA, poly $(A)^+$ RNA and AFP mRNA. The initiation of the chase was effective as isotope incorporation into RNA was stopped by 10 min. The total RNA population showed a long half life at each day of gestation, as was expected since total RNA is mostly rRNA. There was a slight reduction in stability at day 17.5. This is likely due to rRNA constituting a smaller proportion of the total RNA population at day 17.5 than at earlier days of gestation as rRNA synthesis was low at day 17.5.

The decay of poly $(A)^+$ RNA showed the presence of two components of different stability at day 11.5 and 15.5, although this behaviour is not quite as obvious at day 15.5. Similar results have been found in other systems (127). The half life of day 17.5 poly $(A)^+$ RNA appeared to have only one component and was approximately double that of day 11.5 and 15.5. This indicated that mRNA of high stability was synthesized at late stages of gestation. The increase in stable mRNA's other than AFP could account for the decrease in relative synthesis of AFP near birth.

To determine the half life of AFP mRNA, the filter hybridization assay was used. To show that this assay was specific for AFP mRNA, several control experiments were done. The results show that yolk sac total RNA would bind to the pAF-7 DNA but not to blank or chicken DNA. Furthermore radioactive RNA from a non-AFP producing cell line would not bind these filters. The melting point (Tm) of the filter bound hybrid was 88°C (personal communication, G. Andrews, University of Calgary), indicating that there was a great deal of sequence homology between the RNA and DNA (140). We therefore concluded that the assay was detecting only AFP mRNA sequences.

The results of the hybridization experiments showed that the half life of AFP mRNA was very long. An accurate determination of the half life was not possible as the yolk sacs could not be reliably cultured for longer than 30 hr. Accurate analysis was further hampered by the degree of variation in the assay. Therefore lines which best represented the decay of AFP mRNA were drawn and extended to obtain values for the half life. The half life obtained for AFP mRNA was greater than three days. There is no apparent change in AFP mRNA stability during gestation. Therefore the decrease in AFP synthesis near birth is not due to a destabilization of the mRNA which has been found in various inducible systems (169, 127). The half life of AFP mRNA in hepatoma cells was found to be 40 hr (97). Although this value is lower than that found in the yolk sac, in both cases, this message is far more stable than the other mRNA's found in these two cell types.

The stability of AFP mRNA must be important for the changes in relative amount of AFP mRNA during gestation.

CONCLUSIONS

The yolk sac surrounds the fetus from day 9.5 to term. During this period there is a general decrease in the synthesis of DNA, RNA, and protein with a concomitant increase in the relative synthesis of a small group of secreted proteins. These results provide biochemical evidence that the yolk sac is going through a process of terminal differentiation.

AFP is the major protein synthesized and secreted by the yolk sac. The level of AFP synthesis is apparently regulated by the proportion of AFP mRNA. The great stability of AFP mRNA plays an important role in regulating the level of AFP mRNA in the yolk sac during gestation.

AFP is the major secretory protein of the yolk sac. The inhibition of AFP glycosylation by tunicamycin reduced the microheterogeneity of AFP but did not affect AFP secretion. This indicated that glycosylation is not required for AFP secretion.

The yolk sac, therefore, appears to be a useful system to study the regulation of terminal differentiation, AFP gene expression and AFP secretion.

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